Association of the Nucleic Acid of Squash Leaf Curl Geminivirus with the Whitefly *Bemisia tabaci*

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ABSTRACT

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Squash leaf curl geminivirus (SLCV) was detected in its whitefly vector, *Bemisia tabaci*, using full-length cDNA clones of the SLCV genome in a nucleic acid spot hybridization assay. Viral DNA was detected more readily in individual whiteflies ground in buffer and spotted onto membranes than in whiteflies squashed onto membranes. Amounts of viral DNA from 3.2 to 926 pg were detected in individual whiteflies reared on SLCV-infected squash plants. No significant differences were found in the detection rates (number of insects with detectable amount of viral nucleic acid/number of insects tested) of male and female adults, but male insects contained more viral DNA/μg of body weight than females. Viral nucleic acid was detected infrequently in immature stages of *B. tabaci* that developed on infected plants, and this corresponded with low virus transmission rates by adults emerging from tested populations of immature whiteflies. Viral nucleic acid was detected in adult whiteflies for at least 120 hr after 12- and 24-hr acquisition access periods on infected plants. Detection rates and mean amounts of detectable viral nucleic acid remained constant over the 120-hr period, while the whiteflies fed on a nonhost plant. Viral nucleic acid was detected in *Trialeurodes vaporariorum*, a nonvector whitefly species. Longer acquisition access periods resulted in higher rates of detection in adults of *B. tabaci* and in increased rates of transmission. SLCV coat protein was detected in individual adults of *B. tabaci* using an enzyme-linked immunosorbent assay (ELISA). Detection rates, either by ELISA or nucleic acid spot hybridization, did not necessarily reflect transmission rates of individual whiteflies.

Additional keywords: insect transmission, plant virus.

Squash leaf curl is a disease of autumn-planted cucurbits that occurs in the Imperial County of California (4,10,13,14,28). This disease appears to be caused by at least two biologically distinguishable variants of squash leaf curl geminivirus (SLCV) known as SLCV-1 or SLCV, and SLCV-2, melon leaf curl virus or watermelon curly mottle virus (24,32). These variants are not distinguishable by serology but do differ in host range (32). SLCV is transmitted by the sweet potato whitefly, *Bemisia tabaci* (Gennadius), which can occur in high population densities in the Imperial Valley in the fall (12,29, T. M. Perring, *unpublished data*).

The whitefly-transmitted geminiviruses possess a small circular single-stranded DNA genome encapsidated in a geminate particle (reviewed by 18 and 23). Detection of these viruses is complicated because they infect mostly phloem and phloem-associated parenchyma cells, thereby occurring in low concentrations in plant hosts. In addition, the coat proteins of whitefly-transmitted geminiviruses share an extensive amount of sequence homology (35).

The whitefly-transmitted geminiviruses appear to be transmitted in a circulative, persistent manner (5,9,11,18). Geminiviruses are not transmitted transovarially in their vector. However, some can be acquired by the sessile immature stages and transmitted by adults emerging from those immature whiteflies (8,21,33), while others can be acquired and transmitted only by adults (22). Female adults have been shown to transmit geminiviruses more efficiently than males (6,8,34).

SLCV can be transmitted with a minimum acquisition access period of 30 min, a minimum latent period of 8 hr, and a minimum inoculation access period of 30 min (4). Acquisition of SLCV has been shown to reduce the life-span of the vector (4).

Geminiviral coat protein and nucleic acid have been detected in whiteflies by both ELISA and nucleic acid-based assays, respectively (5,9). The use of nucleic acid-based assays has become an acceptable method for detection of viruses and viroids in plant tissues (19,30); however, these assays have not been used to study virus-vector relationships. Therefore we initiated this study to examine the transmission characteristics of SLCV-2 by its vector, *B. tabaci*, using a nucleic acid-based assay.

MATERIALS AND METHODS

Sources of virus, probe DNA, and whiteflies. Cloned geminiviral DNA was obtained from S. Lazarowitz, Carnegie Institute of Washington, Baltimore, MD. Full-length viral genome components were cloned separately in pEMBL plasmids and maintained in *Escherichia coli* 71-18 (25,32). Plasmid DNAs, containing viral inserts, were extracted using an alkali lysis, cesium chloride purification (7).

The geminivirus culture used in this study, SLCV-2, was established in squash, *Cucurbita pepo* L., in 1981 from insects collected in Imperial County, CA (32). The virus culture was maintained in squash by whitefly transmission and in bean, *Phaseolus vulgaris* L. 'Topcrop' by mechanical transmission. *B. tabaci* used in the experiments were obtained from cultures started with insects collected from Imperial County, CA, and maintained on sweet potato (*Ipomoea batatas* L.), cotton (*Gossypium hirsutum* L.), or *P. vulgaris*. Adults of *Trialeurodes vaporariorum* (Westwood) were obtained from a culture started from individuals collected in a greenhouse and were maintained on *Nicotiana tabacum* L.

Virus purification. Virus was purified from mechanically inoculated Topcrop plants (16). Plants were inoculated 7–10 days after planting, maintained in a growth chamber (Environmental model M31-15, Chagrin Falls, OH) at 32 C, with a 14-hr day length, and harvested 12–14 days after inoculation.

Preparation of samples. Plant and insect samples were prepared for blotting according to the procedure of Robinson et al (36). Samples of plant tissue were ground to a powder in a mortar in the presence of liquid nitrogen and then 4.0 ml of TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0) was added for each 1.0 g of tissue. Homogenate was strained through Miracloth. Whiteflies were placed in a 1.5-ml microfuge tube containing 0.025 ml of TE buffer. The pointed end of a 0.4-ml microfuge tube was used to grind the insect. Samples were blotted immediately after processing. Whiteflies to be squashed onto membranes were placed onto the membrane with forceps and were mashed using the rounded end of a glass rod.

Procedure for nucleic acid spot hybridization. Samples were spotted in 0.025-ml aliquots onto Zeta-Probe (Bio-Rad) or Transfor Nylon 66 Plus (Hoefer Scientific, San Francisco, CA) membrane prewetted in TAE buffer (0.04 M Tris-HCl, 0.02 M Na acetate, 0.001M EDTA, pH 7.8) with a blotting manifold and vacuum pressure (Bio-Rad Laboratories, Richmond, CA). After spotting, the membranes were baked for 2 hr at 80 C in a vacuum oven, and either prehybridized immediately or sealed in a plastic bag and refrigerated until hybridization. Blots of insect tissue were prehybridized in 5× SSC (0.75 M sodium chloride, 0.075 M sodium citrate), 50% formamide, 10× Denhardt's solution, 0.1% SDS, 250 μg/ml denatured salmon sperm DNA, and 0.025 mM sodium phosphate buffer, pH 6.5 (Zeta-Probe directions, 26). Plastic boxes containing 0.15 ml of fluid per square centimeter of membrane, were used for prehybridization and hybridization.

The nucleic acid probe used in all experiments was a mixture of equal parts of all four full-length cloned viral DNA components, described in a previous report (32). Probe DNA, consisting of the plasmid plus viral insert, was labeled by nick translation with alpha ^{32}P CTP as described previously (32). The specific activity of labeled probe DNA was approximately 1.0×10^8 CPM/μg of DNA. Labeled probe was boiled for 5 min and added to the membrane in prehybridization fluid to a final concentration of $0.7–3 \times 10^6$ cpm. Membranes were hybridized at 42 C for 24 hr.

Blots normally were prehybridized and hybridized in groups (up to a total of 800 cm^2 membrane). After hybridization groups of blots were rinsed at 55 C twice in 500 ml of 4× SSC, 0.1% SDS, and then four times in 500 ml of 2× SSC, 0.1% SDS. Blots were air-dried and then exposed to X-Ray film (Kodak XR-1) with intensifying screens at −20 C for 4–48 hr (blots of plant tissues) and 48–84 hr (blots of insect tissue).

Procedure for SLCV ELISA. Antisera to SLCV was obtained from J. Duffus (U.S. Agricultural Research Stn., Salinas, CA). Gamma globulin was purified by chromatography on DE 22 cellulose and conjugated with alkaline phosphatase. Double antibody sandwich ELISA was performed using conditions previously reported (32). Whitefly samples were prepared by grinding in a manner similar to that used for nucleic acid spot hybridization in 0.2 ml of PBS-Tween per insect. Measurements of absorbance at 405 nm were made with a Bio-tek EIA reader (Model EL 307, Bio-tek Instruments, Inc., Burlington, VT). Reactions were considered positive when the A_{405nm} values were greater than the mean of the negative controls (four to six whiteflies not exposed to virus, ground individually, and assayed in separate wells on each ELISA plate) plus three times the standard deviation (17,37).

Procedure for whitefly manipulation. Whiteflies were transferred with glass tipped aspirators. All transmission studies involving single insects used whole plant cages to cover the test plants during the inoculation periods. These cages were made of a plastic circular base attached to a Reemay (spunbonded polyester by E. I. Dupont de Nemours & Co. Inc.) cloth top. Transmission studies were conducted year-round in greenhouses at 25–35 C. Whiteflies were given access to virus-infected plants housed in wood-framed and nylon-netted cages large enough to contain several plants. Details of specific experiments are described in the Results section.

Analysis of autoradiograph data. Samples were considered positive when the spot on the autoradiograph was darker than the negative controls. Quantitative measurement of reactions was obtained by using either the maximum absorbance of light at 560 nm or the area under the absorbance peak (mm^2) as determined by a densitometer (Beckman DU-50 spectrophotometer with a Gel Scan Accessory, Palo Alto, CA). Maximum absorbance of spots was determined using the following settings: 1.0 mm horizontal slit width, 0.2 mm vertical slit width, and 10 readings per millimeter.

RESULTS

Detection and quantitation of SLCV nucleic acid in whiteflies. Squashing whiteflies directly onto membranes was compared to grinding and spotting for sensitivity and speed. Grinding of insects yielded a significantly greater frequency of detection than squashing (Table 1). In addition the spots created by ground and spotted insects were more uniform in shape and larger in size than the squashed insects and results were easier to read visually and with a densitometer (Fig. 1). Grinding insects was more time con-

TABLE 1. Results of two methods of extraction for the detection of squash leaf curl virus 2 nucleic acid in individual adult *Bemisia tabaci* fed on virus-infected plants by nucleic acid spot hybridization

Replicate	Treatment[a]	No. Positive /total	Percent[b] positive
1	Squashed[c]	10/24	41.7*
	Ground[d]	19/24	79.2
2	Squashed	9/40	22.5**
	Ground	32/40	80.0

[a] Twenty-two adult whiteflies not exposed to virus were tested by either squashing or grinding and none were found to be positive.
[b] Significant differences between treatments determined by the chi-square test of independence, * $P = 0.05$, ** $P = 0.01$.
[c] Each insect was squashed directly onto the nylon membrane using a glass rod.
[d] Each insect was ground in 25 μl of buffer and homogenate was spotted onto a nylon membrane using a blotting manifold.

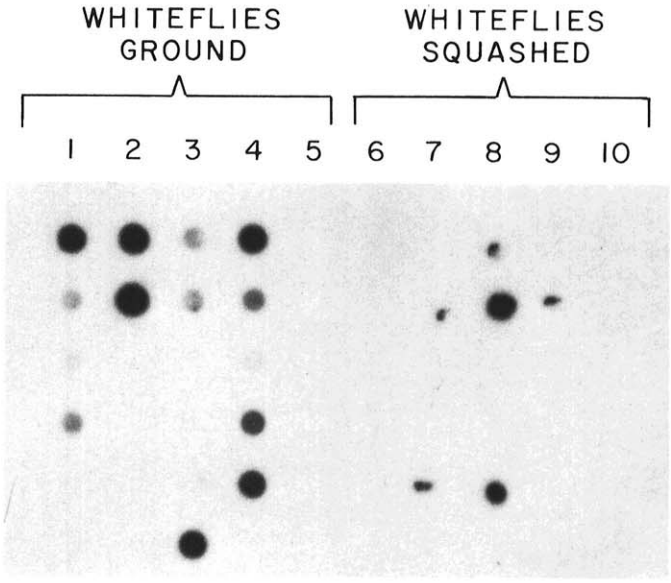

Fig. 1. Autoradiograph of a nucleic acid spot hybridization assay of individual adult *Bemisia tabaci* either ground in TE buffer (0.01 M Tris-HCL, 0.001 M EDTA, pH 8.0) and spotted onto Nylon 66 membrane (24 whiteflies in columns 1 through 5) or squashed directly onto membrane (24 whiteflies in columns 6 through 10). Whiteflies in columns 1 through 4 and 6 through 9 were given a 24-hr acquisition access period on SLCV-2 infected squash plants, and those in columns 5 and 10 were given access to healthy squash plants. SLCV-2 nucleic acid was detected with ^{32}P-labeled cloned viral DNA (described fully in Materials and Methods section). Blots were hybridized to probe DNA as described in Materials and Methods section and exposed to autoradiograph film for 16 hr at −20 C.

suming, but, because of these results, it was the method used for all other experiments.

Two measurements, maximum absorbance at 560 nm and area under the absorbance peak at 560 nm, determined by a DU-50 spectrophotometer, were compared for their usefulness in determining the amounts of nucleic acid detected in individual whiteflies. These two measurements were evaluated with known amounts of viral nucleic acid present in purified virus (0.356 mg/ml) in a twofold serial dilution. The area under the absorbance peak gave a linear response over a wider range of nucleic acid concentrations than the maximum absorbance (Fig. 2), but results were affected more strongly by the calibration site, the choice of smoothing function, and the choice of minimum peak height than were those of the maximum absorbance. The maximum absorbance measurement was superior to the area under the absorbance peak when low concentrations of nucleic acid were detected, since under these situations (when the maximum absorbance approaches the minimum peak height setting) the spectrophotometer's calculation of the area under the absorbance peak became noticeably less accurate. Maximum absorbance appeared to be the better measurement for estimating amounts of nucleic acid in whiteflies, while the area under the absorbance peak would be better suited to estimate concentrations in plant tissue.

Purified SLCV-2 was diluted in twofold series with TE buffer and with a whitefly homogenate made of nonviruliferous adults of *B. tabaci* ground in TE buffer at a concentration of one insect per 0.025 ml to determine if whitefly tissue interferes with the detection of SLCV-2 nucleic acid. The two dilution series were spotted in triplicate and were hybridized at the same time. The maximum absorbance at 560 nm of autoradiograph spots was calculated and standard curves of each dilution series were constructed. No differences in detection of SLCV-2 nucleic acid were observed, indicating that whitefly tissue did not interfere with viral nucleic acid detection. However, with long exposures (80–100 hr), some nonspecific binding of probe to extracts from nonviruliferous whiteflies was seen.

To determine the amount of viral nucleic acid present in the insects, whiteflies were ground in buffer, spotted, and hybridized concurrently with a membrane containing a twofold dilution series of purified virus. Forty adults of *B. tabaci* were chosen at random from a population reared on SLCV-2 infected squash plants. Results were read by a densitometer and the maximum absorbance at 560 nm of each reaction was determined. Viral DNA was detected in 32 of the 40 insects (80%) tested. The range of viral DNA content in whiteflies was estimated at 3.2–926 pg, with a 3.2-pg limit of detection (Fig. 3). The mean viral nucleic acid content was 147.8 pg and the median 20–30 pg. This represents a mean of approximately 4,478 ng of viral nucleic acid per gram of *B. tabaci*, assuming a mean insect weight of 3.3×10^{-5} g (3).

Differences in detection between male and female whiteflies. Adults chosen at random from a colony of *B. tabaci* were given a 24- (experiment 1) or 48-hr (experiment 2) acquisition access period to SLCV-2 infected squash plants and then collected and frozen. Whiteflies were kept chilled over ice, until they were sexed. Twenty-five males and 25 females in experiment 1, and 19 males and females in experiment 2 were ground and spotted onto membranes. The maximum absorbance at 560 nm was determined for insects in each experiment with an autoradiograph of a 14-hr exposure in experiment 1 and 142 hr for experiment 2. Visual assessment and maximum absorbance at 560 nm were compared for determining positive and negative reactions. No significant differences ($P > 0.05$) were found between the qualitative detection of viral nucleic acid in males and females in experiments 1 and 2 (chi-square test of homogeneity). There was no significant

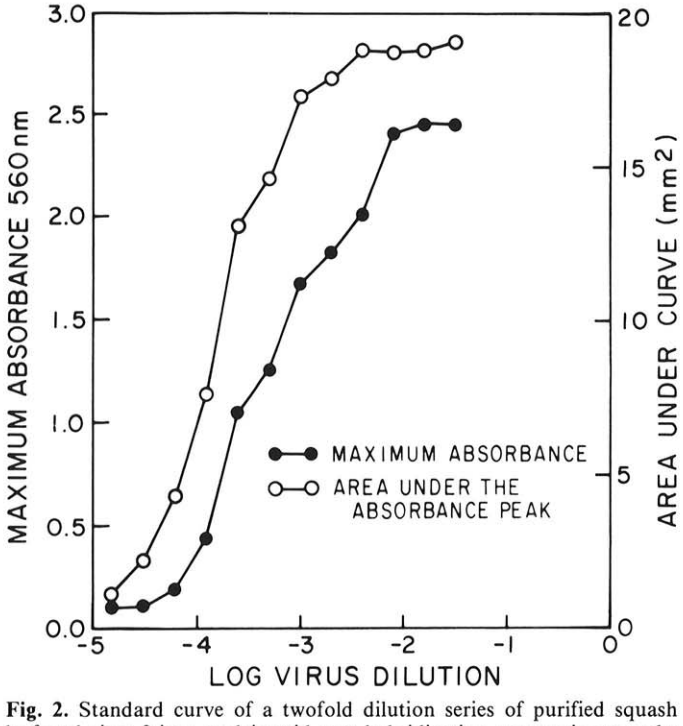

Fig. 2. Standard curve of a twofold dilution series of purified squash leaf curl virus 2 in a nucleic acid spot hybridization assay using a probe composed of ^{32}P-labeled cloned viral DNA and hybridized at 42 C (described fully in Materials and Methods section). The starting concentration of purified virus was 0.356 mg/ml. The concentration of virus at log dilution −1.47 was 2,430 ng, log dilution −2.0, 750 ng; log dilution −3.0, 72 ng; and log dilution −4.0, 7.5 ng. The standard curve was determined by either maximum absorbance at 560 nm or the area under the absorbance peak at 560 nm for an autoradiograph of a 38-hr exposure at −20 C. Each point represents the mean of three values.

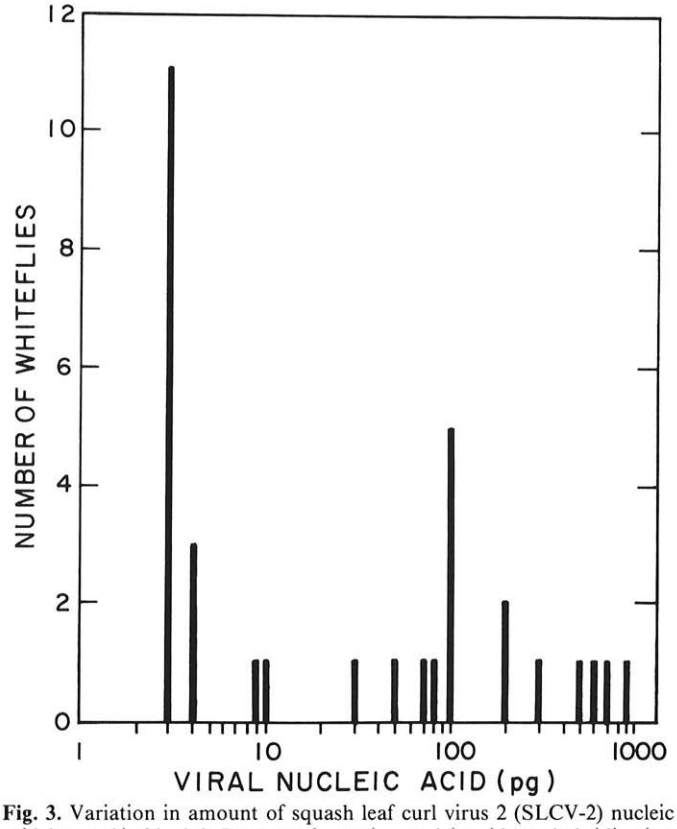

Fig. 3. Variation in amount of squash leaf curl virus 2 (SLCV-2) nucleic acid detected in 31 adult *Bemisia tabaci* using nucleic acid spot hybridization assay with ^{32}P-labeled cloned viral DNA (described fully in Materials and Methods section). Whiteflies were chosen at random from a population raised on SLCV-2 infected squash plants, ground in TE buffer, and spotted onto nylon membranes. Amount of viral nucleic acid (pg) in each insect was determined by comparing the maximum absorbance at 560 nm of each whitefly spot with those of a dilution series of purified SLCV-2 as expressed in a standard curve. Blots were hybridized to probe DNA as described in Materials and Methods section and were exposed to autoradiograph film for 110 hr at −20 C.

difference in the mean A_{560nm} values between male and female whiteflies in experiment 1. However, in experiment 2 the average female whitefly contained significantly more viral DNA than males. The difference in mean A_{560nm} values was significant at $P > 0.05$ when the mean of all males and the mean of all females were compared, and at $P > 0.1$ when the mean of only the males and females rated positive (by A_{560nm} values) were compared. To compare amount of viral DNA per microgram of body weight, the mean absorbance unit/μg of insect body weight was calculated using a mean body weight of 25 μg/male insect and 35 μg/female insect (3). Although statistics could not be performed because individual body weights could not be determined for each insect, there was a trend in both experiments for higher amounts of DNA/μg body weight in male whiteflies.

Detection of viral nucleic acid in immature *B. tabaci*. Detection of viral nucleic acid in immature stages was studied by allowing a large number of whiteflies (not exposed to virus) to deposit eggs on SLCV-2 infected Topcrop plants. Immature stages were detached from the infected plants and were macerated individually or in groups. Some immatures were allowed to develop into adults and these teneral adults were removed with a camel's hair brush from the infected host plant before they fed. These adults were assayed individually for the presence of viral nucleic acid. Leaves containing fourth instar immatures were removed from virus-infected plants and allowed to dry at room temperature. Groups of five to 16, 10–30, and 40–50 adults from these leaves were collected as they emerged and were placed on uninoculated Topcrop plants to determine ability to acquire virus while immature and transmit as adults. Test plants were harvested 2–3 wk after the introduction of the whiteflies and tested by nucleic acid spot hybridization for the presence of virus.

Viral nucleic acid was detected in one of 196 immatures of *B. tabaci* tested individually and in none of 335 immatures tested in groups of five and 10. This was a lower frequency of detection than was found in adults given the same length acquisition access periods. No viral nucleic acid was detected in 63 newly emerged adults that had developed on virus-infected plants. This low frequency of detection was consistent with a low rate of transmission by the newly emerged adults that had access to viral nucleic acid as immatures (Table 2). Only one out of 20 plants and one out of 15 plants which were inoculated by 30–40 and 40–50 newly emerged adults, respectively, became infected.

TABLE 2. Transmission of squash leaf curl virus 2 by adult *Bemisia tabaci* that developed on SLCV-2 infected plants as immatures but were denied access to virus a adults

Experiment	No. of whiteflies/ plant	No. of infected plants[a]/ total plants
I	5–16	0/16
II	10–30	0/17
III	30–40	1/20
IV	40–50	1/15

[a] Positive transmission of SLCV-2 to plants confirmed by nucleic acid spot hybridization.

Effect of acquisition access period on detection and transmission. Adults of *B. tabaci* were placed on virus-infected Topcrop. Sixty insects each were removed at 12 hr, 24 hr, and 24-hr intervals from 24 to 168 hr. Forty whiteflies at each collection period were frozen for later processing and hybridization and 20 were placed on 7- to 10-day-old Topcrop plants, one insect per plant, to determine transmission ability. Plants were observed for 25 days for symptom expression. The experiment was repeated but without establishing transmissibility.

There was a significant positive curvilinear relationship ($r^2 = 0.994$, $P < 0.0001$) between the length of the acquisition access period and the frequency of detection of viral nucleic acid in single whiteflies (Fig. 4). The frequency of detection increased as the length of acquisition access period increased up to a peak of 120 hr, after which time the frequency of detection leveled off. The increase in detection frequency was paralleled by an increase in the transmission ability of the whiteflies up to 120 hr, after which the transmission ability dropped sharply.

Retention of viral nucleic acid by whiteflies. Whiteflies that had emerged within the same 12-hr period were allowed acquisition to SLCV-2 infected Topcrop plants for periods of 12 or 24 hr. Forty whiteflies were removed from each treatment after the acquisition periods and frozen for later analysis (time 0). The rest were placed on cotton, *G. hirsutum*, a nonhost plant. Groups of 40 whiteflies were removed from each treatment at

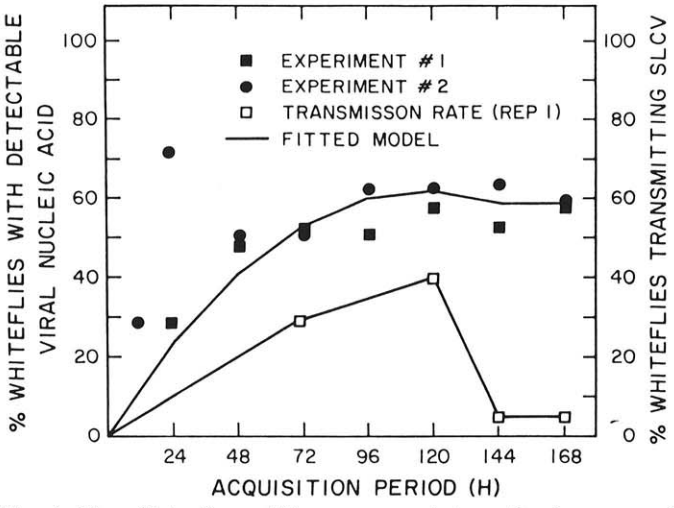

Fig. 4. The effect of acquisition access period on the frequency of transmission and the frequency of detection of squash leaf curl virus 2 (SLCV-2) nucleic acid in individual adult *Bemisia tabaci* using a nucleic acid spot hybridization assay and probe composed of ^{32}P-labeled cloned full-length viral DNA (described fully in Materials and Methods section). Whiteflies were allowed different acquisition access periods on SLCV-2 infected Topcrop, removed, and either placed on healthy Topcrop to determine transmission ability or ground in TE buffer and spotted onto nylon membranes. Blots were hybridized to probe DNA as described in Materials and Methods section and were exposed to autoradiograph film for 90 hr at −20 C.

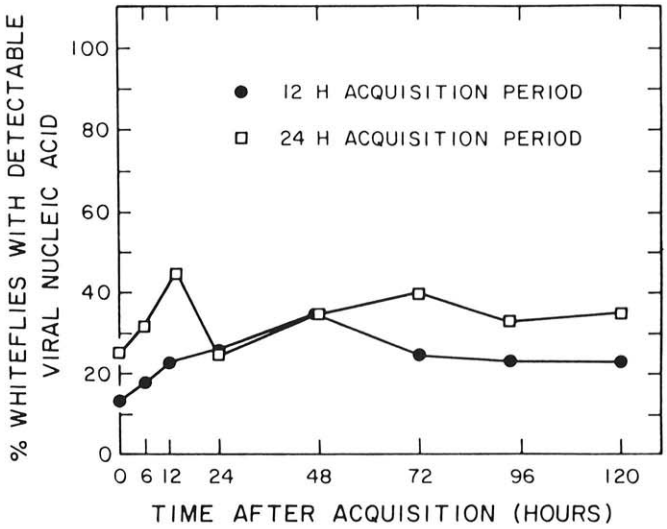

Fig. 5. The detection of squash leaf curl virus 2 nucleic acid over time in individual adult *Bemisia tabaci* after 12- and 24-hr acquisition access periods by nucleic acid spot hybridization using ^{32}P-labeled cloned full-length viral DNA (described fully in Materials and Methods section). Whiteflies, given either a 12- or 24-hr acquisition access period on SLCV-2 infected Topcrop, were placed on cotton and then were removed at various intervals. After removal from cotton, whiteflies were ground in TE buffer and spotted onto nylon membranes. Blots were hybridized to probe DNA as described in Materials and Methods section and exposed to autoradiograph film for 107 hr at −20 C.

intervals of 6, 12, 24, 48, 72, 96, and 120 hr and frozen for later processing and hybridization.

Viral nucleic acid could be detected readily in adults of *B. tabaci* for up to 120 hr after either a 12- or 24-hr acquisition access period (Fig. 5). The frequency of detection following the 12-hr acquisition period was the same or just slightly lower than for 24-hr acquisition period. The detection frequency increased within the first 12–24 hr after acquisition and then remained at a relatively constant level of approximately 25 and 35% for the 12- and 24-hr treatment groups, respectively. The mean A_{560nm} value of each group of 40 whiteflies within each acquisition time remained relatively constant over the 120-hr period. The means for each acquisition period were within one standard deviation of each other in six out of the eight sampling periods, indicating that on the average the amount of nucleic acid within the whiteflies remained constant over the 120-hr period.

Comparison of detection in vector and nonvector species. *T. vaporariorum* and *B. tabaci* adults were placed on virus-infected squash, *Cucurbita pepo* 'Early Prolific'. Forty insects were removed at 24-hr intervals up to 144 hr, frozen, and later ground and spotted onto membranes.

Viral nucleic acid was detected in *T. vaporariorum* adults more often than in *B. tabaci* adults given the same acquisition access periods. The highest rate of detection of SLCV-2 nucleic acid was 100% of individuals of *T. vaporariorum* and 70% of *B. tabaci* individuals when both species were given a 144-hr acquisition access period.

Comparison of detection of nucleic acid and viral coat protein in the whitefly with ability to transmit. Four experiments were conducted in which adult *B. tabaci* were given access to virus-infected squash plants for 24, 48, or 72 hr. After acquisition access periods the whitefly population was divided into two groups. One group was frozen for later analysis by nucleic acid spot hybridization and ELISA. Whiteflies in the other group were placed singly on uninoculated Topcrop plants and allowed to feed for either 24 or 48 hr. At the end of the inoculation access periods live whiteflies were removed and frozen for later analysis by nucleic acid spot hybridization. The Topcrop plants were harvested 3 wk after the inoculation period and were assayed by nucleic acid spot hybridization for the presence of virus.

There was a slight decrease in the detection frequency of viral nucleic acid in adult *B. tabaci* as the inoculation access period increased (Table 3). In general, increases in acquisition access periods resulted in increases in the frequency of detection and transmission. Transmission frequency was not affected by increasing the inoculation access period from 24 to 48 hr.

Viral coat protein was detected in individual whiteflies given acquisition access periods as short as 24 hr (Table 4). Low absorbance values indicated the presence of small amounts of viral antigen in the insects. No significant differences were seen between the rate of detection of SLCV-2 by ELISA in whiteflies allowed 24- and 48-hr acquisition access periods (20–21.4%). There was a higher detection rate (26%) observed in whiteflies allowed a 72-hr acquisition access period.

The rate of detection of SLCV-2 coat protein with ELISA was lower than the detection rate of nucleic acid in individual insects. A better correlation was found between the detection of virus by ELISA and the transmission rate ($r = 0.90$) than between the detection of virus by nucleic acid spot hybridization and the transmission rate ($r = 0.46$). However, the difference between these correlation coefficients was not statistically significant ($0.5 > P > 0.4$) using Student's t test to compare correlation coefficients (38).

Viral nucleic acid was detected in 40 out of 41 adult whiteflies that transmitted SLCV-2. However, viral nucleic acid was detected in 165 out of 219 adult whiteflies that did not transmit virus within a 48-hr inoculation feeding period. Of 54 adult whiteflies in which nucleic acid was not detected, only one transmitted virus. The presence of a detectable amount of viral nucleic acid alone did not appear to be an indicator of the ability to transmit virus under our conditions.

DISCUSSION

Grinding insects in buffer and spotting the extracts onto membrane was superior to squashing insects onto membranes, although others have reported the acceptability of squashing for nucleic acid detection in leafhoppers and *Drosophila* flies (1,2,39). This difference could be due to the small size of whiteflies relative to these other insects, but other factors, such as differences in

TABLE 3. Comparison of the frequency of transmission of squash leaf curl virus 2 (SLCV-2) with the frequency of detection of SLCV-2 nucleic acid in individual adult *Bemisia tabaci* given different acquisition access and inoculation access periods

		0-hr inoculation access period		24-hr inoculation access period			48-hr inoculation access period		
Experiment	Acq[a] Per	Percent positive	No. positive /total	Percent positive	No. positive /total	Percent transmission	Percent positive	No. positive /total	Percent transmission
1	24	74.0	37/50	71.1	32/45	11.1	70.6	36/51	11.8
2	24	77.1	37/48	ns[b]	ns	ns	67.4	31/46	6.5
3	48	95.1	39/41	ns	ns	ns	95.1	39/41	9.8
4	72	91.9	79/86	87.0	33/38	31.5	82.5	33/40	27.5

[a] Acquisition access period in hours.
[b] ns = not sampled.

TABLE 4. Detection of squash leaf curl virus coat protein by double sandwich antibody enzyme-linked immunosorbent assay (ELISA)[a] in individual adults of *Bemisia tabaci* given different acquisition access periods

		Whiteflies exposed to SLCV-2				Whiteflies not exposed to SLCV-2	
				Mean A_{405}[d]			
Experiment	AAP[b]	No. positive /total[c]	Percent positive	Positive	Negative	No. positive /total	Mean A_{405} Negative
I	24	4/20	20.0	0.024 (0.0024)	0.012	0/6	0.013 (0.0017)
II	24	3/14	21.4	0.030 (0.0023)	0.018	0/6	0.008 (0.0058)
III	48	4/20	20.0	0.070 (0.0160)	0.002	0/6	−0.002 (0.0064)
IV	72	9/34	26.4	0.022 (0.0038)	0.013	0/4	0.013 (0.0017)

[a] ELISA procedure used was the one described in the Materials and Methods section.
[b] The acquisition access period in hours.
[c] The number of insects with a positive reaction as determined by the mean of the negative control plus 3 times the standard deviation/total number of insects tested.
[d] The mean absorbance at 405 nm with standard deviation in parentheses of individual adult whiteflies with either positive or negative reactions.

methodology, also could be responsible. This method of extraction was more rapid than the one described by Czosnek et al (9).

We report the detection of 3.2–926 pg of viral DNA per adult *B. tabaci* reared on SLCV-infected squash, with a mean of 147.8 pg of viral DNA per whitefly. This range of viral DNA content may be restricted at the lower end due to the detection limits of the assay. Mean viral DNA content of 4,478 ng/g whitefly was higher than that reported for subterranean clover red leaf virus in aphids (400–600 ng viral RNA/g aphid) reared on virus infected host plants (20). Subterranean clover red leaf virus is semipersistently transmitted in aphids, and *B. tabaci* transmission of geminiviruses has been classified as persistent (11). In maize streak virus transmitting leafhoppers, given a 7-day acquisition access period, the range of viral DNA content per leafhopper was 0.019–1.6 ng, with mean contents of 287 and 382 pg of DNA per male and female adults, respectively (1). No depression or inhibition of detection of viral nucleic acid by whitefly tissue was found. This is similar to results with leafhoppers (1,2).

This is also the first report of the detection of geminiviral coat protein in individual adult whiteflies by ELISA. Virus was detected in approximately 20% of the whiteflies given acquisition access periods of 24–48 hr, and in 26% of the insects given an access period of 72 hr.

Densitometry was an acceptable method to quantitate nucleic acid spot hybridization results. We found this method superior to scintillation counting because of the low levels of radioactivity present on spotted samples of ground whiteflies which we were unable to reliably detect by scintillation counting. Amounts less than 20 pg of maize streak virus could not be detected in leafhoppers by scintillation counting (1). The same restriction was encountered during this study. The maximum absorbance at 560 nm was found to be better than the area under the absorbance peak for quantitating results of detection in insects.

The detection rate of viral nucleic acid in *B. tabaci* was found to depend on the length of the acquisition access period and the morphological stage of the insect. In general, the rate of detection increased as the length of the acquisition access period increased until saturation was reached at 48–96 hr. This positive relationship between acquisition period and rate of detection also was apparent for *T. vaporariorum*, a nonvector whitefly species.

Viral nucleic acid was detected at a higher frequency in adults than in immature stages feeding on similar virus sources. The lack of detection of viral nucleic acid in immature stages coupled with a very low rate of transmission suggests that immatures acquire virus at a lower rate than adults. It is unlikely that immature stages fail to feed in tissue containing high concentrations of virus as often as adults since immature stylets have been measured and shown to be long enough to reach phloem cells (31). Somehow virus is not retained within the immature whitefly body, as it is with adult whiteflies.

The detection rate of viral nucleic acid in *B. tabaci* was found to be independent of sex for up to 48 hr of acquisition feeding period. However, the amount of viral nucleic acid detected per gram of body weight was slightly greater in males than in females given similar acquisition access periods. This was surprising considering that adult female *B. tabaci* have been shown to transmit virus more often and to possess a more sedentary lifestyle, which suggests greater opportunites for feeding (6,8,34). Boulton and Markham (1) found that male leafhoppers had a higher mean concentration of maize streak virus nucleic acid than females when the acquisition access period was less than 4 days; the opposite was observed with acquisition access periods greater than 4 days.

Viral nucleic acid was retained within adults of *B. tabaci* without obvious increase or decrease over a 5-day period, as evidenced by a relatively constant rate of detection and mean A_{560nm} value. This suggests that the viral nucleic acid detected in these insects is not in the lumen of the gut, which is passed in the form of honeydew and fecal matter, but is in the hemocoel or salivary glands, as is the case with aphids and the circulatively transmitted luteoviruses (15). The retention of viral nucleic acid in whiteflies was different than the retention of virus coat protein reported by Cohen et al (5). In contrast to nucleic acid, viral coat protein concentration increased until 24 hr after acquisition and then began a continual decrease.

Viral nucleic acid could be detected in adults of *T. vaporariorum*, a whitefly that does not transmit geminiviruses but is a vector of several closteroviruses (reviewed by 11). These data indicate that *T. vaporariorum* feeds in tissue containing geminivirus DNA and acquires viral DNA in its body but nevertheless is unable to transmit. Surprisingly viral DNA was detected in higher rates in *T. vaporariorum* than in *B. tabaci* and a threshold of detection that appeared with detection of *B. tabaci* was not reached in *T. vaporariorum*. This is similar to results reported on the detection of SLCV by ELISA in groups of *T. abutilonea* and *B. tabaci* (5). Apparently, these nonvector whiteflies do not vector SLCV for reasons other than being able to accumulate viral nucleic acid and antigen.

Detection of viral nucleic acid in individual adults of *B. tabaci* was compared with the ability to transmit SLCV-2. Nucleic acid was detected in whiteflies that transmitted and in whiteflies that did not transmit virus. However, there was a good relationship between the absence of nucleic acid and the failure to transmit. This relationship was observed also in immature whiteflies between the rates of detection and likelihood of transmission as adults.

Nucleic acid spot hybridization and ELISA were compared for their ability to detect virus in adult *B. tabaci* and to predict whitefly transmission rates. The correlation of transmission rate with detection rate was higher with ELISA but changes in the transmission rate were not reflected in this detection rate, suggesting that the correlation was due to the low rates of transmission and ELISA detection, as compared to the high rates of detection by nucleic acid spot hybridization. Neither assay appeared to be a good predictor of the probability of transmission by a single insect. These conclusions of poor correlation between detection of viral antigen by ELISA and transmission are similar to those of Cohen et al (5). Similar studies on the detection of other circulative, nonpropagative viruses in individual insect vectors by ELISA have shown good correlations between detection and transmission rates (27,40).

The results of this study tend to support a persistent, circulative model of the relationship between whiteflies and geminiviruses. Since a nonvector whitefly was shown to accumulate viral nucleic acid to high levels, it is apparent that more than simply ingestion and accumulation within the body is involved in the transmission of squash leaf curl virus.

LITERATURE CITED

1. Boulton, M. I., and Markham, P. G. 1986. The use of squash blotting to detect plant pathogens in insect vectors. Pages 55-69 in: Developments and Applications in Virus Testing. A. C. Jones and L. Torrance, eds. The Lavenham Press Ltd., Lavenham, Sudbury, England.
2. Boulton, M. I., Markham, P. G., and Davies, J. W. 1984. Nucleic acid hybridization techniques for the detection of plant pathogens in insect vectors. Pages 181-186 in: Proc. Br. Crop Prot. Conf. Pests Dis. 1984, Vol. 1. The Administrative Secretary, ed. BCPC Publications, Croydon, England.
3. Byrne, D. N., Buchmann, S. L., and Spangler, H. G. 1988. Relationship between wing loading, wingbeat frequency and body mass in homopterous insects. J. Exp. Biol. 135:9-23.
4. Cohen, S., Duffus, J. E., Larsen, R. C., Liu, H. Y., and Flock, R. A. 1983. Purification, serology and vector relationships of squash leaf curl virus, a whitefly-transmitted geminivirus. Phytopathology 73:1669-1673.
5. Cohen, S., Duffus, J. E., and Liu, H. Y. 1989. Acquisition, interference, and retention of cucurbit leaf curl viruses in whiteflies. Phytopathology 79:109-113.
6. Cohen, S., and Nitzani, F. E. 1966. Transmission and host range of the tomato yellow leaf curl virus. Phytopathology 60:1127-1131.
7. Cooksey, D. A., and Moore, L. W. 1981. Biological control of crown gall with an Agrocin mutant of *Agrobacterium radiobacter*. Phytopathology 72:919-921.
8. Costa, A. S., and Bennett, C. W. 1950. White-fly-transmitted mosaic of *Euphorbia prunifolia*. Phytopathology 40:266-283.

9. Czosnek, H., Ber, R., Navot, N., and Zamir, D. 1988. Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. Plant Dis. 72:949-951.
10. Dodds, J. A., Lee, J. G., Nameth, S. T., and Laemmlen, F. F. 1984. Aphid and whitefly-transmitted cucurbit viruses in Imperial County, California. Phytopathology 74:221-225.
11. Duffus, J. E. 1987. Whitefly transmission of plant viruses. Pages 73-91 in: Current Topics in Vector Research, Vol. 4. K. Harris, ed. Springer-Verlag, New York.
12. Duffus, J. E., and Flock, R. A. 1982. White-fly-transmitted disease complex of the Desert Southwest. Calif. Agric. 36:4-6.
13. Duffus, J. E., Liu, H. Y., and Johns, M. R. 1985. Melon leaf curl virus—A new gemini virus with host and serological variations from squash leaf curl virus. Phytopathology 75:312.
14. Flock, R. A., and Mayhew, D. 1981. Squash leaf curl, a new disease of cucurbits in California. Plant Dis. 65:75-76.
15. Gildow, F. E. 1982. Coated-vesicle transport of luteoviruses through salivary glands of *Myzus persicae*. Phytopathology 72:1289-1296.
16. Goodman, R. M. 1977. Single-stranded DNA genome in a white-fly-transmitted plant virus. Virology 83:171-179.
17. Gordon D. T., Nault, L. R., Gordon, N. H., and Heady, S. E. 1985. Serological detection of corn stunt spiroplasma and maize rayado fino virus in field-collected *Dalbulus* spp. from Mexico. Plant Dis. 69:108-111.
18. Harrison, B. D. 1985. Advances in geminivirus research. Annu. Rev. Phytopathol. 23:55-82.
19. Hull, R. 1984. Rapid diagnosis of plant virus infections by spot hybridization. Trends Biotechnol. 2:88-91.
20. Jayasena, K. W., Randles, J. W., and Barnett, O. W. 1984. Synthesis of a complementary DNA probe specific for detecting subterranean clover red leaf virus in plants and aphids. J. Gen. Virol. 65:109-117.
21. Kirkpatrick, T. W. 1931. Further studies on leaf-curl of cotton in the Sudan. Bull. Entomol. Res. 22:323-363.
22. Laird, E. F., and Dickson, R. C. 1959. Insect transmission of the leaf-crumple virus of cotton. Phytopathology 49:324-327.
23. Lazarowitz, S. 1987. The molecular characterization of gemini-viruses. Plant Molec. Biol. Rep. 4:177-192.
24. Lazarowitz, S. 1990. Molecular characterization of two bipartite geminiviruses causing squash leaf curl disease: Role of transactivation and defective genomic components in determining host range. Virology (In press)
25. Lazarowitz, S., and Lazdins, I. B. 1990. Infectivity and complete nucleotide sequence of the cloned genomic components of SqLCV-E, a bipartite squash leaf curl geminivirus with a broad host range phenotype. Virology (In press)
26. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 545 pp.
27. Mumford, D. L. 1982. Using enzyme-linked immunosorbent assay to identify beet leafhopper populations carrying beet curly top virus. Plant Dis. 66:940-941.
28. Nameth, S. T., Dodds, J. A., Paulus, A. O., and Laemmlen, F. F. 1986. Cucurbit viruses of California: An ever-changing problem. Plant Dis. 70:8-12.
29. Natwick, E. T., and Zalom, F. G. 1984. Surveying sweetpotato whitefly in the Imperial Valley. Calif. Agric. 38:11.
30. Owens, R. A., and Diener, T. O. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-671.
31. Pollard, D. G. 1955. Feeding habits of the cotton white-fly. Ann. Appl. Biol. 43:664-671.
32. Polston, J. E., Dodds, J. A., and Perring, T. M. 1989. Nucleic acid probes for detection and strain discrimination of cucurbit geminiviruses. Phytopathology 79:1123-1127.
33. Rathi, Y. P. S., and Nene, Y. L. 1974. Some aspects of the relationship between mung bean yellow mosaic virus and its vector *Bemisia tabaci*. Indian Phytopathol. 27:459-462.
34. Rathi, Y. P. S., and Nene, Y. L. 1974. Sex of *Bemisia tabaci* Genn. in relation to the transmission of mung bean yellow mosaic virus. Acta Bot. Indica 2:74-76.
35. Roberts, I. M., Robinson, D. J., and Harrison, B. D. 1984. Serological relationships and genome homologies among geminiviruses. J. Gen. Virol. 65:1723-1730.
36. Robinson, D. J., Harrison, B. D., Sequeira, J. C., and Duncan, G. H. 1984. Detection of strains of African cassava mosaic virus by nucleic acid hybridization and some effects of temperature on their multiplication. Ann. Appl. Biol. 105:483-493.
37. Shapiro, S. S., and Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). Biometrika 52:591-611.
38. Snedecor, G. W., and Cochran, W. G. 1967. Statistical Methods. Iowa State University Press, Ames.
39. Tchen, P., Anxolabehere, D., Nouaud, D., and Periquet, G. 1985. Hybridization on squashed flies: A method to detect gene sequences in individual *Drosophila*. Anal. Biochem. 150:415-420.
40. Torrance, L. 1987. Use of enzyme amplification in an ELISA to increase sensitivity of detection of barley yellow dwarf virus in oats and in individual vector species. J. Virol. Meth. 15:131-138.