Fate of Viruses in Bean Leaves After Deposition by *Epilachna varivestis*, a Beetle Vector of Plant Viruses

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ABSTRACT

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Fluorescent antibody labeling was used to detect plant viruses within bean leaves after virus was deposited by Mexican bean beetles previously fed purified virus. Two to three days post feeding, southern bean mosaic virus (SBMV) and bean pod mottle virus, two beetle-transmissible viruses, were detected in veins leading from the feeding wound, and primary infection sites occurred close to feeding wound sites in mesophyll cells associated with these veins. In addition, SBMV antigen and infection sites were detected at some distance from the feeding wound. The location of infection sites for both viruses was confirmed by viruliferous beetle feeding on local lesion hosts. Two non-beetle-transmissible viruses, tobacco ringspot virus and tobacco mosaic virus, were detected only on the edges of feeding wounds at 2–3 days after feeding. At 4–12 h after feeding, all four viruses were found in veins leading from the feeding site, although the non-beetle-transmissible viruses were found in fewer veins and were not detected far from the feeding wound. These results suggest that non-beetle-transmissible viruses introduced by beetle feeding are translocated in veins but to a lesser extent than beetle-transmissible viruses.

In plant cells, injury is known to be required for the establishment of virus infection. This wounding is often accomplished in nature by vectors such as aphids, nematodes, and whiteflies that introduce virus with their stylets directly into cell cytoplasm, where virus uncoating and replication begin (6).

It was once assumed that beetle transmission of viruses occurs through a simple mechanical process involving contamination of beetle mouthparts (9). More recently, however, a more complex type of interaction has been identified. Beetles acquire and deposit both beetle-transmissible and non-beetle-transmissible viruses in regurgitant during feeding (3). Although some of these non-beetle-transmissible viruses, such as the legume isolate of tobacco mosaic virus (CP-TMV) and tobacco ringspot virus (TRSV), are very stable and infectious when delivered to plant leaves on beetle feeding wounds, they fail to induce infection in the assay plants (3).

It has been shown that a ribonuclease (RNase) present in beetle regurgitant selectively inhibits the infection of viruses that are not beetle transmissible when these viruses are deposited by leaf-feeding beetles (5). Gross-wound inoculation, a method that produces damage similar to beetle feeding, has been used to study the effect of beetle regurgitant and RNase in virus transmission (2,4,8). Beetle-transmissible viruses are transmitted by gross-wounding when mixed with beetle regurgitant or RNase, whereas non-beetle-transmissible viruses are not transmitted by gross-wound inoculation in the presence of regurgitant or RNase. It has been suggested that after virus deposition on the leaf in regurgitant, beetle-transmissible viruses escape the effects of RNase by translocating away from wound sites through the xylem and infecting unwounded cells (3). Translocation of southern bean mosaic (SBMV) and bean pod mottle (BPMV) viruses in plant stems after injection of viruses below steamed-stem sections resulted in infection of nonwounded tissue in bean leaves located above virus inoculation sites (1). Additionally, when sodium azide was added to the gross-wound inoculum mixture to produce a halo of dead cells around the gross-wound site of inoculation, the beetle-transmissible SBMV caused infection (3). This suggests that SBMV moves away from wound sites and establishes infection at sites distant from the inoculation wound. The above findings suggest that beetle-transmissible viruses may have a unique ability to translocate in plants following introduction at severely damaged sites and to establish infection in unwounded cells and that this phenomenon is an important determinant in beetle transmission of plant viruses.

The objectives of this investigation were to observe the location of beetle-transmissible and non-beetle-transmissible viruses deposited in wound sites after beetle feeding and to locate virus infection sites in leaves after transmission by using virus-specific immunofluorescent antibody labeling.

MATERIALS AND METHODS

Viruses and virus purification. Two beetle-transmissible viruses (SBMV and BPMV) and two non-beetle-transmissible viruses (CP-TMV and TRSV) were used. SBMV, BPMV, and CP-TMV were propagated in *Phaseolus vulgaris* L. 'Black Valentine' and TRSV was propagated in *Cucumis sativus* L. 'Boston Pickling'. All viruses were purified by the methods described by Gergerich et al (4). After the final high-speed centrifugation, virus was suspended in 0.01 M phosphate buffer (pH 7.2), except BPMV, which was suspended in 0.1 M phosphate buffer (pH 7.2).

Beetles and beetle transmission. Mexican bean beetles (*Epilachna varivestis* Mulsant) were reared in the greenhouse on healthy *P. vulgaris* cv. Pinto plants. Adult beetles were selected 6–11 days after emergence from pupae and were starved for 24 h prior to acquisition feeding. Beetles acquired virus by the "drop-drink" procedure, in which individual beetles were fed purified virus (11–20 mg/ml) in 0.01 M phosphate buffer (pH 7.2) containing 5% sucrose, with the exception of BPMV, which was in 0.1 M phosphate buffer (pH 7.2). Sucrose was added to stimulate beetles to feed on drops. Beetles acquired virus during a 24-h acquisition period from 50-μl drops of the virus mixture placed in individual plastic petri dishes. Negative controls consisted of a 50-μl drop of sucrose/buffer mixture without virus. Beetles that were observed feeding on the drops were transferred to the primary leaves

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of individual Black Valentine bean plants in which the first trifoliolate leaves had not emerged. After feeding (two or more wounds per leaf), beetles were removed and plants were placed under a bank of fluorescent lights in the laboratory for 4–12 or 48–72 h before being processed for virus localization. Controls were Black Valentine bean plants at the primary leaf stage that were dusted with Carborundum and mechanically inoculated with purified virus (1 mg/ml). In similar experiments, transmission rates after drop-drink beetle feeding were determined by placing test plants in the greenhouse for 2 wk and assaying for virus infection by gel double-diffusion tests.

For SBMV and BPMV, Pinto bean, a local lesion host, was also utilized for virus transmission tests using drop-drink beetle acquisition of purified virus in sucrose or sucrose alone as described above. After beetle removal, plants were placed under a bank of fluorescent lights in the laboratory and examined twice daily for lesion development. Controls consisted of Pinto bean plants at the primary leaf stage that were dusted with Carborundum and mechanically inoculated with purified virus (1 mg/ml) or 0.01 M phosphate buffer (pH 7.2); 0.1 M phosphate buffer (pH 7.2) was used for BPMV. Necrotic areas on plant leaves were tested for the presence of virus by cutting out the necrotic area, grinding it with a glass rod in 50 μl of 0.01 M phosphate buffer (pH 7.2; 0.1 M phosphate buffer for BPMV, pH 7.2), and inoculating Carborundum-dusted half leaves of Pinto bean. Samples from nonnecrotic areas of feeding wounds were also tested for virus as a negative control. If lesion counts on half leaves inoculated with samples from necrotic areas were higher than lesion counts of samples from nonnecrotic areas, the necrotic area was considered virus-induced and not the result of mechanical damage.

Preparation of antibody. To monitor virus antigen distribution, indirect immunofluorescent labeling was carried out utilizing the immunoglobulin (Ig) fraction of rabbit antisera obtained by ammonium sulfate precipitation. Antibodies specific for plant antigens were removed from polyclonal virus antisera by mixing one volume of partially purified plant protein (prepared by the same method as described for virus purification from Black Valentine bean leaves) with three volumes of antiserum and incubating for a minimum of 1 h at 37 C, followed by incubation at 4 C for 24–48 h. After low-speed centrifugation at 4,300 *g* for 10 min, an equal volume of a saturated ammonium sulfate solution was added to the supernatant and held at room temperature for 10–15 min before centrifugation at 4,300 *g* for 10 min. The precipitated Ig fraction was washed with 50% saturated ammonium sulfate and centrifuged at 4,300 *g* for 10 min. The final precipitate was resuspended in 0.01 M phosphate-buffered saline (PBS) (pH 7.2), dialyzed against two changes of PBS, and stored frozen or at 4 C in 0.02% sodium azide. Prior to addition of sodium azide, protein concentration was determined spectrophotometrically at 280 nm.

Indirect immunofluorescent labeling. A modification of the procedure described by Lei and Agrios (7) was used in this study. After bean plants had been inoculated (either mechanically or by beetle feeding) followed by an appropriate incubation period, leaves were detached from the main stem and the lower epidermis of each leaf was brushed with an artist's watercolor paintbrush (1/2-in. Sceptre No. 606, Winsor and Newton, England) that had been dipped in a slurry of Carborundum in distilled water. Gentle, uniform brushing (50–100 strokes) was continued until the Carborundum slurry did not appear to bead on the lower epidermis and the leaf surface lost some of its waxy appearance. The leaves were washed with distilled water to remove the Carborundum. Depending on the size of the leaf, whole leaves or leaf pieces were processed. The pieces of larger test leaves were cut with a razor blade so that the midvein bordered one side to give added stability. Beetle feeding wounds were selected to represent different parts of the leaf, including the leaf edge, the area between the leaf edge and the midrib, and the area adjacent to the midrib.

The leaves or leaf pieces were placed brushed side down in small glass petri dishes containing an enzyme solution (2% cellulase and 1% Driselase [Sigma Chemical Co., St. Louis, MO], 0.5 M mannitol, and 10 mM $CaCl_2$, pH 5.5) and incubated 30–60 min on a rotary shaker (20 rpm) at room temperature. More brush strokes and longer incubation times were necessary for older leaves to promote adequate leaf digestion. After enzyme treatment, the leaf pieces were washed with two changes of PBS at 5-min intervals. Specimens were decolorized and fixed in acetone. The time required for decolorization varied with the age of the leaf and ranged from 2 h for young leaves to 24 h for old leaves. After acetone removal, specimens were washed in two changes of PBS at 30-min intervals. Specimens were incubated in virus-specific antibody (38 μg/ml) for 18–24 h at room temperature on a rotary shaker, washed twice with PBS, and incubated for 18–24 h at room temperature on a rotary shaker with fluorescein isothiocyanate-conjugated goat antirabbit antiserum (Sigma) diluted 1:320 in PBS. Leaf specimens were washed, mounted in 10% glycerol in PBS on glass slides (5 × 7.5 cm), and examined with an epifluorescent microscope (490-nm excitation filter, 455-nm accessory edge filter, and 515-nm barrier filter; Model BHT, Olympus Optical Instruments Co., Tokyo, Japan). Photographs were taken with Kodak ED 200 color slide film.

RESULTS

The Mexican bean beetle transmitted SBMV (57/60 plants) and BPMV (48/60 plants) but not CP-TMV (0/30 plants) or TRSV (0/30 plants) to Black Valentine bean after drop-drink acquisition from purified virus preparations. The non-beetle-transmissible CP-TMV and TRSV were not transmitted after drop-drink acquisition, even though higher concentrations of virus were present in the drops than are normally found in plant leaves infected with these viruses.

A bright-green, virus-specific fluorescence was detected in clusters of infected leaf mesophyll cells of Black Valentine bean 2–3 days after mechanical inoculation with all four viruses used in this study (Fig. 1A). The fluorescence was located in the interior of the cell and was most intense around the cell periphery. This fluorescence was not observed in noninoculated leaves or in inoculated leaves treated with preimmune serum instead of virus-specific antiserum. A nonspecific, bright-yellow fluorescence was observed in the foot cells of trichomes in all treatments. Depending on the extent of decolorization, varying amounts of nonspecific red (Fig. 1B) or dull-green fluorescence was observed in some leaves that had not decolorized completely.

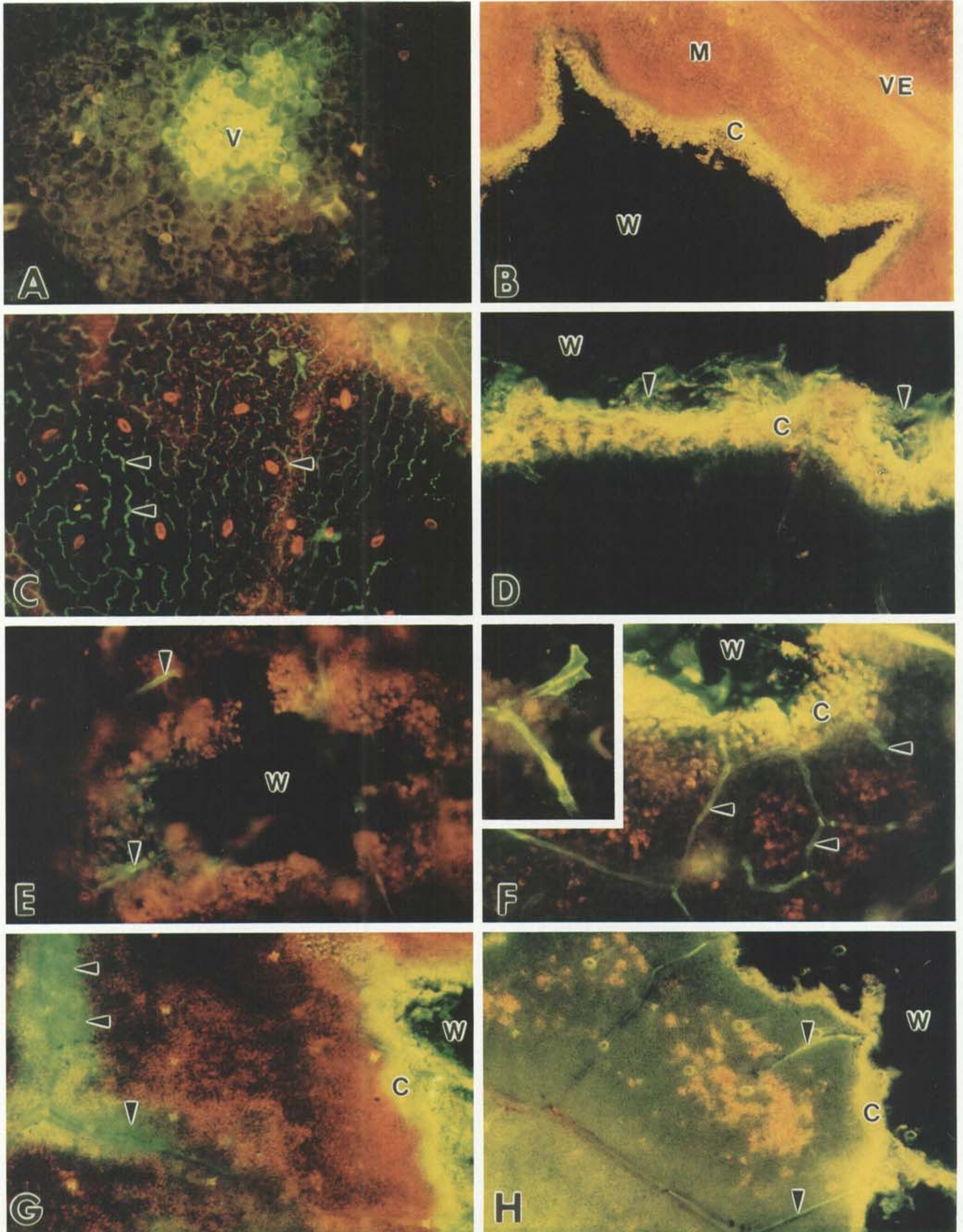
Feeding wounds produced by nonviruliferous beetles were examined for nonspecific fluorescence. In wounds that were 2–3 days old, a yellow-orange fluorescence was associated with the wound-closing layer on the edge of the feeding wound (Fig. 1B). This wound-closing layer was absent or incompletely formed in feeding wounds that were processed 4–12 h after beetle feeding. For the purpose of accurately describing feeding wounds, the area where the beetle damaged and removed leaf tissue will be designated as the wound side and the area beyond the wound-closing layer will be designated as the intact side.

Virus antigen was detectable in and around the feeding wounds made by beetles that had acquired virus by drop-drink feeding after 4–12 h and 2–3 days (Fig. 1D–H). Residual virus was detected in anticlinal grooves of the epidermal surface close to the feeding wound (Fig. 1C). Residual virus antigen was also detected on the wound side of the wound-closing layer for all of the viruses used in this study (Fig. 1D). Beetles produced wounds on all parts of the primary leaves of bean but avoided the midrib and large veins.

Non-beetle-transmissible viruses. Antigens of TRSV and CP-TMV were detected in close association with the wound site 4–12 h after beetle feeding. Occasionally, TRSV was detected inside veins leading from the beetle feeding wound (Fig. 1E), while CP-TMV was detected inside veins associated with a wound site in only two of 22 beetle feeding wounds (Table 1). Leaves processed 2–3 days after beetle feeding contained no detectable TRSV or CP-TMV antigen beyond the wound-closing layer, although virus-specific fluorescence was sometimes observed on

the wound side of the wound-closing layer (Fig. 1D).

Beetle-transmissible viruses. SBMV was detected on the intact side of the wound and in veins directly associated with the wound 4–12 h after beetle feeding (Fig. 1F). Residual virus antigen was also observed in the wound side of the wound-closing layer. Intense fluorescence was detected in many veins leading from wound sites, producing a "road map" effect of virus antigen presence (Fig. 1F). Intense fluorescence was also observed in terminal tracheids, some of which were far from a wound site, 4–12 h after feeding (Fig. 1F inset). Leaves processed 2–3 days after beetle feeding contained



localized SBMV-specific fluorescence in the mesophyll cells and in veins associated with the wound. In addition, areas of fluorescing mesophyll cells located distant from wound sites were detected adjacent to veins (Fig. 1G).

BPMV was also detected in mesophyll cells beyond the wound-closing layer and in veins leading from wound sites 4–12 h after beetle feeding (Fig. 1H). These findings were similar to those with SBMV, but fewer fluorescing veins were observed around wound sites with BPMV, and SBMV translocated further into the veins. Two to three days after beetle feeding, areas of fluorescing mesophyll cells were predominantly detected adjacent to the wound. Fluorescing mesophyll cells associated with veins were occasionally detected several millimeters from wound edges.

Local lesion response in Pinto bean after feeding by viruliferous beetles. Leaves containing wounds caused by beetles that had acquired SBMV or BPMV by drop-drink feeding began developing observable lesions 2–3 days after beetle feeding. SBMV-induced lesions were located within 2 mm of wound edges in 10 of 21 wounds. However, SBMV-induced lesions were also located more than 2 mm away from the wound edge either directly on veins or closely associated with veins in five of 21 wounds.

BPMV-induced lesions were detected in four of 19 leaves at beetle-induced wound sites. All lesions were associated with veins and were very close (<2 mm) to the wound edge.

DISCUSSION

Primary infection sites of mechanically inoculated viruses were readily detected in the mesophyll cells of whole bean leaves by indirect, virus-specific, immunofluorescent antibody labeling. Both beetle-transmissible and non-beetle-transmissible viruses were deposited at feeding wound sites, as evidenced by virus-specific fluorescence on the edges of the feeding wound, confirming earlier reports that non-beetle-transmissible viruses are acquired and deposited on leaves in beetle regurgitant (3).

The major difference between beetle-transmissible and non-beetle-transmissible viruses was the location of the beetle-delivered viruses 2–3 days after feeding. The non-beetle-transmissible viruses were found only on the wound side of the wound-closing layer, and virus-specific fluorescence was not present in veins leading from the wound site. In contrast, the beetle-transmissible viruses were found in numerous veins leading from the wound site, in mesophyll cells associated with the veins on the intact side of the wound-closing layer, and on the wound side of the wound-closing layer. These results 2–3 days after feeding suggest either that the non-beetle-transmissible viruses were not readily translocated into the veins following feeding or that they were translocated but degraded in the leaf (Fig. 1E, Table 1).

At 4–12 h after beetle feeding, the four viruses used in this study reacted differently from one another when observed by means of virus-specific fluorescent-antibody labeling. The non-beetle-transmissible viruses, TRSV and CP-TMV, were observed only occasionally in short segments of veins near the wound site. The beetle-transmissible BPMV was also somewhat restricted to mesophyll and short segments of veins near the wound site, but many more fluorescing veins were observed around wounds caused by beetles that had acquired BPMV than around wounds caused by beetles that had acquired the non-beetle-transmissible viruses (Table 1). The results for non-beetle-transmissible viruses at 4–12 h after feeding are in contrast to the observations at 2–3 days after feeding in that these viruses were not found in the veins leading from the wound site at 2–3 days after feeding. This suggests that the non-beetle-transmissible viruses may be degraded following translocation into short segments of the veins leading from the wound edge.

The present study demonstrated that SBMV moved in veins further from the feeding wound than any of the other viruses used and that infection by SBMV occurred in unwounded cells away from the edge of the feeding wound. The transmission study using Pinto bean, a local lesion host for SBMV and BPMV, confirmed that SBMV indeed infects unwounded cells, since local lesions were formed away from the edge of the feeding wound. In contrast, indirect immunofluorescent microscopy showed that BPMV did not translocate very far into veins leading from the feeding site and that infection loci were close to the feeding wound. Beetle transmission of BPMV to Pinto bean confirmed that local lesions were formed only adjacent to the wound edge. These results demonstrate that BPMV and SBMV, two beetle-transmissible viruses, differ in their ability to translocate in plants. However, from these experiments it is unclear whether the BPMV that is delivered by beetles in regurgitant on feeding wounds is infecting wounded cells on the edge of the feeding site or unwounded cells a short distance from the wound site.

Beetles deposit virus in regurgitant on the edges of feeding wounds, and regurgitant contains RNase (2), a powerful inhibitor of virus infection (5). Therefore, it seems reasonable to assume that the cells exposed to RNase on the edge of beetle feeding wounds are not susceptible to virus infection because of the codeposition of virus and RNase. Since SBMV appears to readily translocate to unwounded cells far away from the wound site through veins of the leaf, it is clear that this virus escapes the effects of RNase in the regurgitant. On the other hand, BPMV behaved similarly to the two non-beetle-transmissible viruses in that it appeared to move only short distances in leaf veins, although BPMV invaded more leaf veins than the non-beetle-transmissible TRSV and CP-TMV. Perhaps the non-beetle-transmissible viruses that move into the xylem either are unstable in the xylem or lack the ability to pass from the xylem to other tissues, thus becoming confined to the wound area where they are ultimately degraded.

TABLE 1. Virus-specific fluorescence in leaf wounds after deposition of viruses by the Mexican bean beetle

Virus[a]	Hours after feeding 4–12	48–72
Non-beetle-transmissible		
TRSV	8/20[b]	0/25
CP-TMV	2/22	0/23
Beetle-transmissible		
SBMV	34/40	27/30
BPMV	33/35	30/46

[a] Beetles acquired virus from drops of purified virus (15–20 mg/ml) in 5% sucrose, then were given access to primary leaves of Black Valentine bean. Leaves with feeding wounds were processed at different times after feeding for immunolocalization of virus.

[b] Number of wound sites containing fluorescing veins or mesophyll/number of wounds processed for immunolocalization.

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Fig. 1. Micrographs of the abaxial side of Black Valentine bean leaves tested for virus antigen by indirect fluorescent antibody labeling: **A,** Infection locus (V) of fluorescing mesophyll cells in a leaf mechanically inoculated with southern bean mosaic virus (SBMV) 2–3 days after inoculation (×300). **B,** Beetle-induced feeding wound area (W), wound-closing layer (C), and nonspecific fluorescence of red-orange mesophyll cells (M) and vein (VE) in leaf tissue 2–3 days after nonviruliferous beetle feeding (×120). **C,** Leaf surface showing SBMV-specific fluorescence near feeding wounds in the anticlinal grooves (arrowheads) of epidermal cells 2–3 days after beetle feeding (×300). **D–H,** Beetle-induced feeding wounds (W) with bright-green, virus-specific fluorescence (arrowheads). **D,** Tobacco mosaic virus antigen (arrowheads) on the edge of the wound-closing layer (C) 2–3 days after beetle feeding (×300). **E,** Tobacco ringspot virus antigen (arrowheads) inside veins leading from the feeding wound (W) 4–12 h after beetle feeding (×300). **F,** SBMV antigen (arrowheads) in veins leading from wound sites 4–12 h after beetle feeding (×300). (Inset) Intense fluorescence in terminal tracheids (×420). **G,** SBMV antigen (arrowheads) in mesophyll cells directly associated with veins distant from the wound site (W) 2–3 days after beetle feeding; C = wound-closing layer (×120). **H,** Bean pod mottle virus antigen (arrowheads) in veins leading from the feeding wound (W) 4–12 h after beetle feeding; C = wound-closing layer (×120).

Two important questions regarding the interaction of beetle-transmissible viruses with plants still remain to be answered. The first is in regard to the interaction of viral and plant factors that regulate the translocation of viruses in plants, and the second is related to how viruses in the xylem infect unwounded cells.

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