Vector Relations

Noncirculative Transmission of Plant Viruses by Leaf-Feeding Beetles

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ABSTRACT

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The movement of four plant viruses into the hemocoel of chrysomelid and coccinellid beetle vectors after ingestion was studied. None of the four plant viruses (two beetle-transmissible and two non-beetle-transmissible) were detected in the hemolymph of the Mexican bean beetle (*Epilachna varivestis*), an efficient plant virus vector in the family Coccinellidae, regardless of the acquisition source, type of virus, or method of virus detection. The infectivity of viruses was not destroyed by the hemolymph of the Mexican bean beetle, as demonstrated by virus survival in hemolymph for up to 3 days after virus injection into the hemocoel. Only one beetle-transmissible and one non-beetle-transmissible virus tested were found in the hemocoel of the bean leaf beetle (*Cerotoma trifurcata*) and the spotted cucumber beetle (*Diabrotica undecimpunctata howardi*), both members of the family Chrysomelidae. These results indicate that virus movement into the beetle hemocoel is determined by the nature of the interaction between the individual virus and beetle, and that some plant viruses that are noncirculative, such as bean pod mottle comovirus, can be efficiently transmitted by beetle vectors.

Additional keywords: cowpea strain of tobacco mosaic virus, southern bean mosaic virus, tobacco ringspot virus.

Beetles have long been known to be vectors of four groups of plant viruses (6,18). Unlike most plant virus–vector relationships, the transmission of plant viruses by leaf-feeding beetles is determined by the interaction of the virus with the host plant as well as by the interaction of the virus with the vector (7). Even though both beetle-transmissible and non-beetle-transmissible viruses are delivered by the beetle in regurgitant during feeding, only some of these viruses successfully infect the host plant (8). It has been shown that ribonuclease in beetle regurgitant prevents infection by non-beetle-transmissible viruses when these viruses are deposited in regurgitant on the leaf surface during feeding (9).

Although beetle-transmissible and non-beetle-transmissible viruses that are acquired by beetle feeding on virus-infected leaves are found in the regurgitant of beetle vectors (15), it is not known whether the viruses found in regurgitant are simply regurgitated from the foregut (a foregut-borne type of relationship), or whether they must move into the hemolymph prior to their appearance in the regurgitant (a circulative type of relationship). Freitag (2) recovered squash mosaic comovirus from the hemolymph of western striped cucumber beetles, *Acalymma trivittata* (Mann.), and western spotted cucumber beetles, *Diabrotica undecimpunctata undecimpunctata* Mann., after virus acquisition from infected tissue. Since then, other viruses, such as cowpea mosaic comovirus (16), the cowpea strain of tobacco mosaic tobamovirus (15), the cowpea strain of southern bean mosaic sobemovirus (13,17), and southern bean mosaic sobemovirus (15) have been recorded in the hemolymph of beetle vectors. The hemolymph of the bean leaf beetle, *Cerotoma trifurcata* (Forster), was shown to be a reservoir for the cowpea strain of southern bean mosaic virus, and beetles were viruliferous after the injection of purified virus into the hemocoel (14,15). Ingested southern bean mosaic virus (cowpea strain) appeared very rapidly in the hemolymph of bean leaf beetles (13). The concept that beetle-transmitted viruses are present in the hemolymph of their vectors has been widely accepted (5–7,19), and it has been hypothesized that movement into the hemolymph plays a role in the transmission process (4,6,17,20).

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The purpose of this research was to investigate the movement of beetle-transmissible and non-beetle-transmissible viruses into the hemolymph of different species of beetle vectors and to determine the importance of this circulation in the virus transmission process.

MATERIALS AND METHODS

Viruses. Two beetle-transmissible viruses (southern bean mosaic sobemovirus [SBMV] and bean pod mottle comovirus [BPMV]) and two non-beetle-transmissible viruses (the cowpea strain of tobacco mosaic tobamovirus [CP-TMV] and tobacco ringspot nepovirus [TRSV]) were used in this study. Previous research has shown that SBMV and CP-TMV are present in the hemolymph of bean leaf beetles after acquisition from infected plants (15), but BPMV and TRSV are not (16). These are stable, easily purified, mechanically transmissible viruses that cause systemic infection in bean (*Phaseolus vulgaris* L. 'Black Valentine'). SBMV and BPMV are transmitted efficiently by the three species of beetles used in this study (3,5,6). All viruses were propagated in 'Black Valentine' bean, except TRSV, which was propagated in cucumber (*Cucumis sativus* L. 'Boston Pickling') in the greenhouse. The viruses were purified by the methods described by Gergerich et al (8).

Beetles. Adults and larvae of the Mexican bean beetle (*Epilachna varivestis* Muls.) were reared in a greenhouse on Pinto bean. The larvae of bean leaf beetles and spotted cucumber beetles (*Diabrotica undecimpunctata howardi* Barber) are root feeders and therefore difficult to rear in the laboratory. During the growing season, adult bean leaf beetles and spotted cucumber beetles were collected in the field from soybean and bean plants and from squash, respectively, and maintained on detached, healthy primary leaves of Pinto bean that were changed daily for more than 1 wk before the beetles were used. During the winter, spotted cucumber beetles were purchased from French Agricultural Research Inc. (Lamberton, MN).

Virus acquisition from infected plants. Individual beetles were given an acquisition access to virus-infected leaves in a petri dish in the laboratory at room temperature for 1 or 24 h. Each beetle was watched individually to determine the beginning of the acquisition period. For those beetle-virus combinations, from which no infectious viruses were recovered from the hemolymph 24 h after acquisition, the acquisition time was prolonged by feeding the beetles on virus-infected plants in cages continuously for 1 wk.

Drop-drink experiments with purified virus. Purified viruses (10–20 mg/ml) were mixed with an equal volume of 20% sucrose in 0.01 M phosphate buffer (BPMV in 0.1 M phosphate buffer), pH 7.2. Beetles were starved for 24 h and then individually allowed access for 1 or 24 h to drops of this virus solution. Only beetles that were observed to feed on the virus solution were used in experiments.

Injections. Each beetle was injected with 50–70 μg of purified virus in 5 μl of 0.01 M phosphate buffer (BPMV in 0.1 M phosphate buffer), pH 7.2. Virus was injected into the hemocoel between the third and the fourth abdominal segments dorsally with a microapplicator (model M, Instrumentation Specialties Co., Lincoln, NE), using a syringe with a 32-gauge hypodermic needle. Beetles were fed on healthy Pinto beans in the interval between injection and hemolymph collection.

Recovery of virus from the hemolymph. Mexican bean beetle larvae were held with fingers, and one of the metathoracic legs was severed with a pair of scissors. For hemolymph collection from all adult beetles, beetles were confined on a fabricated stage, and one of the metathoracic legs was removed with a pair of forceps. The resulting drop of hemolymph was collected with a capillary tube, diluted 100- to 200-fold with 0.01 M phosphate buffer (BPMV with 0.1 M phosphate buffer), pH 7.2, and inoculated on Carborundum-dusted leaves of the appropriate local lesion host. The results of all hemolymph assays were the pooled results of at least two experiments.

Virus detection. The local lesion host used for virus detection of SBMV and BPMV was *P. vulgaris* L. 'Pinto'; for CP-TMV, *Chenopodium quinoa* Willd.; and for TRSV, *C. sativus*. Bean plants were inoculated when the primary leaves were fully expanded; *C. quinoa* was inoculated before blooming; and cucumber was inoculated at the cotyledon stage.

Enzyme-linked immunosorbent assay. Protein A sandwich enzyme-linked immunosorbent assay (PAS-ELISA) was used to measure virus titer in beetle hemolymph (1). Mexican bean beetles and spotted cucumber beetles were starved for 24 h, then fed detached virus-infected Black Valentine bean leaves individually in petri dishes for an acquisition period of 24 h. After feeding, the hemolymph was collected individually as described above and diluted 50- to 100-fold in phosphate-buffered saline (0.02 M phosphate and 0.15 M NaCl, pH 7.4) containing 0.05% Tween 20. The hemolymph of eight beetles fed healthy bean plants was used as a negative control, and the hemolymph of eight beetles injected with purified virus (5 μg of purified virus in 5 μl of 0.01 M phosphate buffer, pH 7.2) was used as a positive control. Quantitative measurements of the alkaline phosphatase reactions were made by determining absorbance at A_{405nm} with an MR 300 MicroELISA plate reader (Dynatech Labs, Chantilly, VA). Two times the mean of the healthy absorbance reading plus one standard deviation was established as the threshold of positive reactions.

RESULTS

Movement of virus into the hemocoel after acquisition from infected plants. The movement of ingested plant viruses into the hemocoel of leaf-feeding beetles was dependent on the combination of beetle and virus (Table 1). For example, no infectious

TABLE 1. Recovery of viruses from beetle hemolymph after virus acquisition from infected plants

		Percentage of beetles with virus in hemolymph[a]			
Beetles	Acquisition time[b]	SBMV	BPMV	CP-TMV	TRSV
Mexican bean beetle	1 h	0 (0/12)[c]	0 (0/12)	0 (0/12)	0 (0/14)
	24 h	0 (0/36)	0 (0/24)	0 (0/24)	0 (0/28)
	1 wk	0 (0/27)	0 (0/26)	0 (0/26)	0 (0/26)
Bean leaf beetle	1 h	7 (2/29)	0 (0/30)	9 (3/32)	0 (0/28)
	24 h	13 (5/38)	0 (0/30)	28 (5/18)	0 (0/35)
	1 wk	ND[d]	0 (0/37)	ND	0 (0/42)
Spotted cucumber beetle	1 h	6 (1/18)	0 (0/26)	25 (6/24)	0 (0/24)
	24 h	17 (8/48)	0 (0/26)	24 (8/34)	0 (0/70)
	1 wk	ND	0 (0/42)	ND	0 (0/45)

[a] SBMV = southern bean mosaic sobemovirus; BPMV = bean pod mottle comovirus; CP-TMV = cowpea strain of tobacco mosaic tobamovirus; TRSV = tobacco ringspot nepovirus.

[b] The length of time beetles were fed on infected tissue.

[c] The ratio in parentheses is the number of beetles having virus in the hemolymph/number of beetles tested.

[d] Not determined.

viruses were recovered from the hemolymph of Mexican bean beetles regardless of which virus was tested, even after 1 wk of acquisition feeding. Infectious CP-TMV and SBMV were recovered from the hemolymph of the spotted cucumber beetle and the bean leaf beetle (two species of chrysomelid beetles) after only 1 h of acquisition feeding. No infectious BPMV or TRSV was recovered from the hemolymph of any of the beetles tested.

Since both the adult and the larvae of the Mexican bean beetle transmit plant viruses (4,10), the movement of virus particles within the beetle larvae was also tested. Second- or third-stage larvae of the Mexican bean beetle were fed either SBMV- or CP-TMV–infected leaves for 24 h (50 beetles for each virus individually). After virus acquisition, hemolymph was collected from individual larvae and inoculated on local lesion hosts. Hemolymph from 10 nonviruliferous larvae and from 10 virus-injected larvae was collected and inoculated for the control. No infectious virus was recovered from the hemolymph of beetle larvae that had fed on SBMV- or CP-TMV–infected leaves. The hemolymph collected from all 10 virus-injected larvae contained infectious virus, indicating that 24 h after injection the infectivity of the virus had not been destroyed by the hemolymph.

Movement of virus into the hemocoel after acquisition from drops of purified virus. Because the failure to recover infectious virus from the hemolymph of some beetles may be due to a low virus concentration in the infected leaves, beetles were fed highly concentrated purified viruses and then tested for virus in their hemolymph. No infectious viruses were recovered from the hemolymph of the Mexican bean beetle after a 24-h acquisition period (Table 2). In contrast, SBMV and CP-TMV were recovered from the hemolymph of the bean leaf beetle and the spotted cucumber beetle after only 1 h of feeding.

Stability of plant viruses in the hemocoel of beetles. One possible explanation for the inability to detect viruses in the hemolymph is that the viruses are not stable in the hemolymph or that they are phagocytized and destroyed by hemocytes (11). To test this hypothesis, purified viruses were injected into the hemocoel, and after different time intervals the hemolymph was collected and assayed for virus by inoculation on local lesion hosts.

Both of the two beetle-transmissible and two non-beetle-transmissible viruses retained infectivity in the hemocoels of the three kinds of beetles for up to 3 days (Table 3). The combinations of viruses and beetles differed in the level and time of virus retention. SBMV remained in the hemocoel of all the spotted cucumber beetles for 3 days. In contrast, after the same amount of time the number of spotted cucumber beetles retaining BPMV in their hemolymph was greatly reduced. TRSV remained in the hemocoel of the spotted cucumber beetle for 3 days, but the number of Mexican bean beetles with virus in their hemocoel remained low for all 3 days.

Detection of virus in the hemolymph of beetles using ELISA. The inability to detect TRSV and BPMV in the hemolymph of the three beetles by local lesion assay could be due to the lower infectivity of these multicomponent viruses. Therefore, ELISA was used to detect the presence of the virus protein in the hemolymph. The use of ELISA for detection also circumvented the possibility of inhibition of infection in local lesion assays by hemolymph components (6).

Results of the ELISA tests (Table 4) are in agreement with the results of the infectivity assays. Neither beetle-transmissible nor non-beetle-transmissible viruses were detected in the hemolymph of the Mexican bean beetle. SBMV and CP-TMV, but not BPMV and TRSV, were detected in the hemolymph of the

TABLE 2. Recovery of viruses from beetle hemolymph after acquisition of purified viruses by drop-drink feeding

Beetles	Acquisition time[b]	Percentage of beetles with virus in hemolymph[a]			
		SBMV	BPMV	CP-TMV	TRSV
Mexican bean beetle	1 h	0 (0/22)[c]	0 (0/24)	0 (0/24)	0 (0/28)
	24 h	0 (0/12)	0 (0/14)	0 (0/14)	0 (0/18)
Bean leaf beetle	1 h	13 (4/30)	0 (0/36)	33 (13/39)	0 (0/36)
	24 h	35 (7/20)	0 (0/36)	29 (6/21)	0 (0/33)
Spotted cucumber beetle	1 h	13 (3/24)	0 (0/24)	39 (7/18)	0 (0/32)
	24 h	42 (5/12)	0 (0/18)	42 (21/50)	0 (0/35)

[a] SBMV = southern bean mosaic sobemovirus; BPMV = bean pod mottle comovirus; CP-TMV = cowpea strain of tobacco mosaic tobamovirus; TRSV = tobacco ringspot nepovirus.
[b] The length of time beetles were fed on drops of purified virus (10–20 mg/ml of purified virus was mixed with an equal volume of 20% sucrose).
[c] The ratio in parentheses is the number of beetles having virus in the hemolymph/number of beetles tested.

TABLE 3. Recovery of viruses from beetle hemolymph after injection of purified viruses into the hemocoel

Beetles	Time after injection[b]	Percentage of beetles with virus in hemolymph[a]			
		SBMV	BPMV	CP-TMV	TRSV
Mexican bean beetle	5 h	100 (18/18)[c]	100 (14/14)	100 (12/12)	3 (1/30)
	24 h	86 (12/14)	100 (14/14)	100 (40/40)	20 (6/30)
	48 h	70 (7/10)	100 (14/14)	78 (28/36)	30 (9/30)
	72 h	28 (5/18)	0 (0/32)	64 (9/14)	20 (6/30)
Bean leaf beetle	5 h	100 (10/10)	100 (18/18)	100 (22/22)	100 (14/14)
	24 h	100 (14/14)	67 (12/18)	100 (20/20)	100 (18/18)
	48 h	100 (18/18)	39 (7/18)	100 (18/18)	100 (21/21)
	72 h	100 (21/21)	50 (9/18)	83 (20/24)	100 (18/18)
Spotted cucumber beetle	5 h	100 (10/10)	100 (12/12)	100 (15/15)	100 (12/12)
	24 h	100 (18/18)	67 (10/15)	100 (13/13)	100 (14/14)
	48 h	100 (16/16)	20 (3/15)	100 (17/17)	100 (18/18)
	72 h	100 (14/14)	17 (3/18)	67 (16/24)	100 (18/18)

[a] SBMV = southern bean mosaic sobemovirus; BPMV = bean pod mottle comovirus; CP-TMV = cowpea strain of tobacco mosaic tobamovirus; TRSV = tobacco ringspot nepovirus.
[b] Interval between virus injection (a dose of 50–70 μg of purified virus in 5 μl of 0.01 M phosphate buffer [BPMV in 0.1 M phosphate buffer], pH 7.2) and hemolymph collection. Beetles were fed on healthy Pinto beans in the interval between injection and hemolymph collection.
[c] The ratio in parentheses is the number of beetles having virus in the hemolymph/number of beetles tested.

spotted cucumber beetle. The ELISA values for hemolymph collected from beetles fed on healthy leaves ranged from 0.10 to 0.16. The ELISA values for SBMV and CP-TMV in the hemolymph of the spotted cucumber beetle showed a bimodal distribution with approximately 20% of the beetles having high ELISA values ranging from 0.46 to 0.58 and 80% having values similar to beetles fed on healthy plants ranging from 0.10 to 0.18.

DISCUSSION

This study indicates that some beetle-transmissible plant viruses are not found in the hemocoel of their beetle vectors and appear to have a foregut-borne or semipersistent type of relationship. Neither beetle-transmissible nor non-beetle-transmissible viruses tested moved into the hemocoel of the Mexican bean beetle, even though this is a very efficient vector of plant viruses (4,10). Similar results were obtained with both adults and larvae of the Mexican bean beetle. Although SBMV and CP-TMV were detected in the hemocoel in two members of the family Chrysomelidae, the bean leaf beetle and the spotted cucumber beetle, neither the non-beetle-transmissible TRSV (a nepovirus) nor the beetle-transmissible comovirus BPMV were detected in the hemolymph of the bean leaf beetle and spotted cucumber beetle, regardless of the acquisition source (infected tissue or purified virus) or the method used to detect the virus (infectivity tests or ELISA). Therefore, the circulative nature of plant viruses depends on the beetle species and appears to be unnecessary for transmission of BPMV by the three beetles in this study. However, another comovirus, squash mosaic virus, has been recovered from the hemolymph of the western striped cucumber beetle (2), spotted cucumber beetle (16) and striped cucumber beetle *Acalymma vittatum* (Fabricius) (16) using infectivity assays of hemolymph from beetles that had fed on virus-infected leaves.

Earlier work showed that viruses (including SBMV and CP-TMV that were used in this study) are found in the hemolymph of beetle vectors such as the bean leaf beetle (13,15–17), spotted cucumber beetle (16), striped cucumber beetle (16), western spotted cucumber beetle (2), and western striped cucumber beetle (2). However, the work done by Slack and Fulton (16) showed that BPMV and TRSV could not be recovered from the hemolymph of the bean leaf beetle after a 24-h acquisition feed on infected host plants. They speculated that the failure to demonstrate BPMV and TRSV in the hemolymph of viruliferous beetles was due to the techniques employed or to the inability of BPMV and TRSV to enter the hemocoel. Later, Fulton and Scott (6) demonstrated that the hemolymph of the bean leaf beetle contained an inhibitor, and that at least a 200-fold dilution of the hemolymph was necessary to overcome the inhibition. The former negative results with TRSV and BPMV were thought to be due to the use of undiluted hemolymph in their tests. The work reported here confirms their inability to detect BPMV and TRSV in the hemocoel of bean leaf beetles and extends their finding to the spotted cucumber beetle and Mexican bean beetle. Therefore, the interaction of plant viruses with beetle vectors appears to be governed by both the virus and the species of beetle.

TABLE 4. Detection of virus in beetle hemolymph by enzyme-linked immunosorbent assay 24 h after virus acquisition from infected leaves

	Percentage of beetles with virus in hemolymph[a]			
Beetles	SBMV	BPMV	CP-TMV	TRSV
Mexican bean beetle	0 (0/56)[b]	0 (0/72)	0 (0/56)	0 (0/64)
Spotted cucumber beetle	21 (14/67)	0 (0/72)	18 (10/56)	0 (0/72)

[a] SBMV = southern bean mosaic sobemovirus; BPMV = bean pod mottle comovirus; CP-TMV = cowpea strain of tobacco mosaic tobamovirus; TRSV = tobacco ringspot nepovirus.

[b] The ratio in parentheses is the number of beetles having positive reaction/number of beetles tested. Two times the mean of the healthy absorbance reading plus one standard deviation was established as the threshold of positive reactions.

Walters et al (20) reported that BPMV can overwinter in hibernating bean leaf beetles, and Slack and Scott (17) demonstrated that virus injected into the hemocoel of the bean leaf beetle can serve as a source of virus for transmission of the cowpea strain of SBMV. The latter results suggested that for longer retention times virus particles might reside in the hemolymph of the beetle. Our results, however, indicate that BPMV does not move into the hemocoel of the bean leaf beetle. We conclude that movement of virus into the hemocoel of the beetle vector is unnecessary for virus transmission or retention by the bean leaf beetle, and that BPMV is retained somewhere in the digestive system during prolonged periods of retention.

SBMV and CP-TMV were recovered from the hemolymph of the bean leaf beetle and spotted cucumber beetle, but the percentages were less than 50% regardless of the acquisition source, type of virus, or method of virus detection. Since intermediate ELISA values were not observed, the ELISA tests for detection of viruses in the hemolymph of the spotted cucumber beetle indicate a qualitative mechanism for virus movement into the hemolymph rather than a quantitative mechanism. The mechanisms regulating movement of plant viruses through the gut wall and the site of transport from the digestive tract to the hemolymph of the insect are unknown.

Some very efficient beetle vectors of plant viruses do not allow movement of viruses into the hemocoel, and even the same beetle selectively allows only some viruses to pass into the hemolymph. The Mexican bean beetle is a very efficient vector of SBMV and BPMV (4), and the bean leaf beetle and spotted cucumber beetle transmit BPMV very efficiently (5). However, in each case, these viruses were not detected in the hemolymph of these beetles. We conclude that although circulation of plant viruses into the hemocoel does occur in some beetle-virus combinations, it is not a necessary prerequisite for beetle transmission of plant viruses.

Although virus transmission by beetles has been previously characterized as a circulative type of vector relationship (3,5,7), the results presented in this paper indicate that some plant viruses that are very efficiently transmitted by beetles do not spread into the hemocoel of their beetle vectors. This suggests a type of virus–vector relationship similar to the "foregut-borne" or semipersistent transmission of plant viruses by leafhoppers (12). Foregut-borne viruses "have no latent period in the vector, cannot be recovered from vector hemolymph, and cannot be transmitted after injection into the vector's hemocoel" (12). The only departure in beetle transmission from this definition of foregut-borne viruses is that virus injected into the hemocoel of beetles results in virus transmission (15,16), even in those virus-beetle combinations where ingested virus does not move into the hemocoel (16). It appears that the relationship of plant viruses with their beetle vectors can be divided into two groups, those in which the viruses are restricted to the digestive system, and those that circulate into the hemocoel of their beetle vectors.

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