EVIDENCE OF MULTIPLICATION OF NORTHERN CEREAL MOSAIC VIRUS IN ITS INSECT VECTORS

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Introduction

The northern cereal mosaic disease was first described in Hokkaido, northern part of Japan, by ITO and FUKUSHI (12). They (13, 14) found inclusion bodies in the cells of the young diseased oat plants and thus concluded that this disease was caused by a virus. Northern cereal mosaic virus (NCMV) was neither transmitted by the diseased juice nor by diseased seeds or soil. Four species of planthoppers were deserved as vectors: *Laodelphax striatellus* FALLÉN (13), *Delphacodes albifascia* MATSUMURA (9, 10), *Unkanodes sapporonus* MATSUMURA (28), and *Muellerianella fairmairei* PERRIS (11).

MURAYAMA and LU (21) studied some physical properties of NCMV by the insect-injection method. Electron microscopic studies by SHIKATA and LU (27) demonstrated large bacilliform particles, 60×300–350 mμ in ultrathin sections of cells of diseased barley plants, and fine threads of 40×500–600 mμ in the cells of infective planthoppers. These particles correspond in size and shape with the virus particles appearing in the infectious fractions of partially purified preparations from both insect and plant hosts (16, 17).

However, there was no experimental evidence on the multiplication of the virus within insect vectors.

The present paper reports the results obtained by serial passages of NCMV from insects to insects. The technique followed the procedure used by MARAMOROSCH (18) for serial passages of the aster yellows virus in leafhopper vectors. The organ culture technique originally developed by HIRUMI and MARAMOROSCH (7) was used to ascertain the multiplication sites of NCMV within the vector.

Materials and Methods

Northern cereal mosaic virus (NCMV) was obtained from stock of diseased

barley plants, *Hordeum vulgare* L. var. HOKUTO HADAKA. Young, healthy barley seedlings were inoculated by planthoppers, *Laodelphax striattellus* FALLÉN. Virus free planthoppers were maintained on rice (*Oryza sativa* L.) plants in a greenhouse insectary. Viruliferous planthoppers were obtained by feeding on diseased barley plants for 2 days, and then confined to rice plants, which are immune to NCMV infection.

The following organs were dissected under a binocular microscope: gut, salivary glands, and fat body. All excised organs were rapidly washed in fresh 0.1 per cent solution of Hyamine 2389 (Rohm and Haas Co., methyldodecylbenzyltrimethylammonium chloride and trimethylammonium chloride) (1, 30) diluted with Ringer-Tyrode solution (3) followed by culture medium. Thirty each of these organs were crushed at 4°C and homogenized in sterilized rough surface hollow slides, in a buffered culture medium. The homogenate was centrifuged in a capillary at 2,000 rpm for 10 minutes at 4°C. The supernatant fluids were injected into 60 healthy, young adult planthoppers by fine glass needles in a cold room at 5°–8°C. Surviving insects were tested infectivity. Other groups of the dissected organs were transferred to culture media. 5 or 6 organs of each kind were maintained in a concave holed slide with 5 drops of culture medium, and sealed with cover glasses, using vaseline. After 10 days' incubation at 25°C, the cultured organs were washed in a fresh culture medium, ground in a hole slide, and then centrifuged. The supernatant fluids were injected into healthy insects by the same procedure. MITSUHASHI's medium (20) was used as the culture medium of these organs.

Injected insects were confined to rice plants for 10 days, then individually tested for infectivity on young barley seedlings. The insects were transferred to healthy test plants every 7 days. All insects were maintained at 25°C during the infective feeding, the incubation period on rice plants, and the transmission tests.

Experimental results

1) Serial passages of NCMV in *L. striatellus* by artificial injections.

The virus source was obtained from 23 nymphs which fed on diseased barley plants for 4 days and maintained for an additional 10 days on rice plants. These insects (0.0023 gr) were macerated, diluted to 1 : 50 with 0.1 M ammonium acetate solution and centrifuged for 10 minutes at 2,000 rpm. After injection of 0.0001 ml of the supernates into each insect, the planthoppers were held for 10 days on rice plants and then transferred individually to barley plants for the following 4 days. Surviving insects of the first groups were macerated, diluted with 1 : 50 and centrifuged at 2,000 rpm for 10 minutes

and then used to inoculate virus free insects. Successive injections were given to four groups of insects.

The original virus had thus been diluted to approximately $1/2 \times 10^{-11}$ after the 4th passage. According to MURAYAMA and LU (21), the dilution end point of NCMV in injected insect vectors is 10^{-4}. Therefore, the results presented in Table I demonstrated that NCMV multiplied in insect vectors.

TABLE I. Results of serial passages of NCMV in *L. striatellus*

Groups of serial passages	Total of injected insects	Survivors after 15 days	Total of infected insects	Fractions of original virus used as source for each dilution series
Original virus source		23	8	
1	60	21	6	$1/2\times10^{-2}$
2	60	12	5	$1/2\times10^{-5}$
3	60	10	2	$1/2\times10^{-8}$
4	60	4	2	$1/2\times10^{-11}$

TABLE II. Results of the tests for the presence of NCMV in extracts from gut, salivary gland and fat body of planthoppers. The organs were dissected following 2 days of aquisition feeding and 1 day of feeding on rice plants, and used as source of inoculum into virus free planthoppers*.

Virus source	Subgroup of insects in cage No.	Development of NCMV on plants exposed to planthoppers at different periods after injection, in days				
		10–16	17–23	24–30	31–37	38–44
Gut	1	− 1**	− 1	− 1	D	
	2	+ 1	+ 1	D		
	3	− 1	+ 1	D		
	4	− 1	− 1	D		
	5	+ 1	D			
	6	− 3	D			
	7	− 3	+ 3	D		
	8	− 3	D			
	9	− 3	D			
	10	− 3	− 2	D		
	11	− 3	+ 1	D		
	12	+ 3	D			
	13	− 3	− 1	D		
	14	− 3	− 1	D		

Virus source	Subgroup of insects in cage No.	Development of NCMV on plants exposed to planthoppers at different periods after injection, in days				
		10–16	17–23	24–30	31–37	38–44
	15	− 4	− 1	D		
	16	− 4	D			
	17	− 4	− 1	D		
Salivary gland	1	d				
	2	− 1	− 1	D		
	3	− 1	D			
	4	− 1	− 1	D		
	5	− 1	− 1	D		
	6	− 1	− 1	− 1	D	
	7	− 1	− 1	D		
	8	− 1	D			
	9	− 1	− 1	− 1	D	
	10	− 1	− 1	+ 1		
	11	− 1	− 1	− 1	D	
	12	− 1	− 1	− 1	D	
	13	− 1	− 1	− 1	− 1	
	14	− 1	− 1	D	− 1	D
	15	− 1	− 1	D		D
	16	− 1	− 1	D		
	17	− 1	D			
	18	− 1	D			
	19	− 1	D			
Fat body	1	− 4	− 3	− 1	D	
	2	− 4	− 2	− 1	d	
	3	− 4	− 3	− 3	D	
	4	− 4	− 2	D		
	5	− 4	− 4	− 2	D	
	6	− 4	− 1	D		
	7	− 4	− 3	− 1	D	
	8	− 4	− 1	− 1	d	
	9	− 4	D			
	10	− 4	− 2	D		
	11	− 4	− 2	− 1	D	

* Virus free planthoppers were injected with extracts from excised organs of 30 insects; after injection the insects were held for 9 days on virus immune plants and then subgroups of not more than 4 planthoppers were transferred serially every week from one virus free plant to another to determine when they would develop capacity to transmit virus.

** + Indicates diseased plant; − healthy plant; numbers indicate the number of

surviving insects in each cage; D indicates last insect had died; d indicates test plant had died.

2) Recovery of NCMV from various organs of viruliferous insect vectors.

Exp. 1 consisted of insects injected with extracts from gut, salivary gland and fat body obtained from insects that had been confined to diseased barley plants for 2 days and to rice plants for 1 day feeding. Forty-four individuals injected with gut extracts survived more than 10 days. They were transferred 4 times during the 37 days following their injection. The transmission results are shown in Table II.

The insects in cage No. 2, 3, 5, 7, 11, 12, which were injected with gut extracts transmitted NCMV consistently after an incubation period of 10 to 23 days.

Of 19 inoculated subgroups, only one insect, in cage No. 10 which was injected with salivary gland extracts, transmitted the virus after an incubation period from 24 to 30 days. This incubation period was considerably longer than that of the virus in the insects injected with gut extracts.

Insects of the other subgroups, injected with extracts from fat body, failed to transmit the virus.

Exp. 2 consisted of insects injected with organs derived from vectors maintained for 2 days on diseased barley plants, and later kept for 10 days on rice plants for the completion of virus incubation in the insects. Virus was recovered from insects injected with extracts from gut, salivary gland, and fat body tissues.

As shown in Table III, the results of the test in which the virus source consisted of gut extracts revealed that 4 subgroups of 10 inoculated subgroups transmitted the virus after an incubation period of 10 to 16 days.

In salivary gland extracts, of 7 inoculated subgroups, 4 subgroups transmitted after an incubation period of 10 to 23 days. The results indicated high virus concentration in the salivary glands.

In fat body tissne extracts, of 12 inoculated subgroups, 4 subgroups transmitted after an incubation period of 10 to 23 days (as shown in Table III).

Exp. 3 consisted of insects injected with extracts from gut, salivary glands, and fat body tissues maintained in vitro for 10 days, following 2-days infective feeding and 1-day of feeding on rice plants. The virus was recovered from extracts of gut and salivary glands but not from fat body. The results are shown in Table IV.

TABLE III. Results of the test for presence of NCMV in gut, salivary gland and fat body of planthoppers by injection into virus free planthoppers. The organs were excised following 2 days of aquisition feeding and 10 days of feeding on rice plants.

Virus source	Subgroup of insects in cage No.	Development of NCMV on plants exposed to planthoppers at different periods after injection, in days			
		10–16	17–23	24–30	31–37
Gut	1	+ 3*	+ 2		
	2	− 3	− 3		
	3	− 3	− 3		
	4	− 3	− 3		
	5	+ 3	+ 3		
	6	+ 3	+ 1		
	7	+ 3	− 1		
	8	− 3	− 1		
	9	− 3	− 2		
	10	− 2	− 2		
Salivary gland	1	− 3	+ 2	+ 2	− 1
	2	+ 3	+ 2	+ 2	+ 1
	3	+ 3	+ 2	+ 1	+ 1
	4	d 3	D		
	5	+ 3	d 1	+ 1	+ 1
	6	d 3	D		
	7	d 3	− 1	− 1	− 1
Fat body	1	d 3	D		
	2	− 3	+ 1	+ 1	D
	3	d 3	− 1	D	
	4	+ 3	D		
	5	d 3	D		
	6	− 3	D		
	7	− 3	d 1	D	
	8	d 3	d 2	+ 1	+ 1
	9	d 3	d 2	D	
	10	d 3	d 2	D	
	11	− 3	− 1		
	12	+ 3	d 2	− 1	− 1

* D and d: See Table II, footnote**.

TABLE IV. Results of the test for the presence of NCMV in gut, salivary gland and fat body of planthoppers in vitro by injection into virus free planthoppers. The dissected organs from insects, which were fed on diseased plants for 2 days and on rice plants for 1 day, were maintained in vitro for the completion of virus incubation, then used as source for inoculum.

Virus Soucre	Subgroup of insects in cage No.	Development of NCMV on plants exposed to planthoppers at different periods after injection, in days				
		10-16	17-23	24-30	31-37	38-44
Gut	1	d 3*	− 1	D		
	2	d 3	D			
	3	d 3	D			
	4	d 3	− 1	D		
	5	+ 3	+ 1	+ 1	+ 1	D
	6	d 3	D			
	7	d 3	D			
	8	− 3	+ 1	D		
	9	+ 3	+ 3	d 2	D	
	10	+ 3	d 3	+ 1	d 1	D
	11	d 3	D			
	12	d 3	D			
	13	d 3	− 1	− 1	D	
	14	d 3	D			
	15	+ 3	− 1	D		
	16	− 3	− 1	d 1	D	
	17	− 3	D			
Salivary gland	1	− 3	− 2	D		
	2	− 3	− 1	D		
	3	+ 3	− 2	− 2		
	4	d 3	D			
	5	− 3	− 2	D		
	6	− 3	− 2	− 1	− 1	D
	7	− 3	− 2	− 1		
	8	− 3	− 2	D		
	9	− 3	+ 2	− 1	− 1	− 1
	10	− 3	D			

Virus source	Subgroup of insects in case No.	Development of NCMV on plants exposed to planthoppers at different periods after injection, in days				
		10–16	17–23	24–30	31–37	38–44
Fat body	1	− 3	− 3	− 2	D	
	2	− 3	− 3	− 2	− 1	− 1
	3	− 3	− 3	D		
	4	− 3	− 3	− 3	− 2	− 1
	5	− 3	− 3	− 3	− 2	− 1
	6	− 3	− 3	− 1	− 1	D
	7	− 3	− 3	D		
	8	− 3	− 3	− 3	D	
	9	− 3	− 3	− 2	D	
	10	− 3	− 2	− 1	− 1	D
	11	− 3	D			
	12	− 3	D			
	13	− 3	− 1	D		

*, D and d: See Table II, footnote**.

Of 17 inoculated subgroups, 5 subgroups transmitted the virus from gut extracts after an incubation period of 10 to 23 days.

Of 10 inoculated subgroups, 2 subgroups transmitted the virus from salivary gland extracts after an incubation period of 10 to 23 days, as shown in Table IV. No. virus was found upon injection of extracts from fat body tissues.

Discussion

Direct evidence for multiplication of certain plant viruses in their leafhopper vectors has accmulated from experiments on serial passage in insect vectors by artificial injection (2, 15, 18, 19). The results of the present experiments showed that NCMV passed in series from vectors to vectors, without decrease in virus concentration, while the insects kept immune plants. The dilution ent point of NCMV was reported between 10^{-3} and 10^{-4} in extracts from viruliferous insects (21). However, when the original virus extract had been diluted to approximately $1/2 \times 10^{-11}$ after the 4th passage, there was no evidence of decrease in infectivity of the insects. Thus it was shown that NCMV multiplied in *L. striatellus* insect vectors. Some polyhedral plant viruses have been found in gut, salivary glands and other organs of their vectors. Rice dwarf virus particles were present in the cells of fat body,

hemolymph, intestinal epithelium, salivary glands, ovarial tubules and Malpighian tubules (4, 5, 24). Wound tumor virus was demonstrated in epidermal cells, mycetome, fat body, muscle, Malpighian tubules, trachea, gut and salivary gland (22, 23), the nervous system (8) and the hemolymph (6, 25). SINHA and BLACK (29) employed the precipitin ring test to show that wound tumor virus antigens could be detected in the fat body tissues, salivary glands, intestine and ovaries of viruliferous leafhoppers, *Agallia constricta* VAN DUZEE.

The results of the presence of NCMV in various organs are summarized in Table V.

TABLE V. A summarized table of the experiments shown in Table II–IV.

Treatment of insects after 2 days acquisition		Recovery of the virus from various organs		
Expt. No.	Treatment during incubation period	Gut	Salivary glands	Fat body tissues
1	Organs excised immediately, extracted, then injected after 1 day of feeding on rice	10–23* ++(6/17)**	24–30 +(1/19)	—
2	Organs excised after 10 days of feeding on rice plants, extracted, then injected	10–16 ++(4/10)	10–23 +++(4/7)	10–23 ++(3/12)
3	After 1 day of feeding on rice plants, organs excised immediately, maintained in vitro 10 days, extracted, then injected	10–23 ++(5/17)	10–23 +(2/10)	—

* Incubation period (in days) in insects injected with tissue extract.
** Numerator: number infected plants.
Denominator: number of test plants.

The virus acquired at the initial 2 days' feeding reached the gut after at least 3 days, and was recovered at higher concentrations after 12 days. Only minute amounts of virus were recovered from salivary glands after 3 days, with considerably longer incubation periods, whereas a high concentration was reached in salivary glands within 12 days in the insects. The high recovery of the virus from salivary glands indicated that the virus gradually accumulated or multiplied in this organ, where it apparently had been stored in sufficient amounts to permit its transmission into susceptible plants. In addition, the recovery of the virus from gut and salivary glands which were maintained in culture media for 10 days showed that NCMV is still present at almost similar concentrations in the gut or at higher concentrations in salivary glands than these in the organs during the initial 3 days of feeding.

All the results mentioned above indicate that NCMV might multiply in the gut, to a limitted extend, and sufficiently multiply in the salivary gland of the insect vectors, *L. striatellus*.

SHIKATA and MARAMOROSCH (22, 26) suggested on the basis of electron microscopic observations that fat bodies are the most likely site of wound tumor virus multiplication in insect vectors. However, the NCMV was recovered from fat body tissues only after 12 days in the insects, but none was recovered after 3 days or after 10 days in vitro, although a few particles of the virus reached the salivary gland after 3 days.

The results suggests that the fat body tissues of *L. striatellus* ars not active sites of virus multiplication of NCMV.

Summary

This paper deals with NCMV multiplication in its insect vector, *Laodelphax striatellus* FALLÉN and the site of virus multiplication within the vector.

In a serial passage by vector injection, the virus was diluted to approximately $1/2 \times 10^{-11}$ after the 4th passage and there was no evidence of decrease in virus titer, as measured by insectivity of the insects.

Attempts were made to recover the virus from excised gut, salivary glands and fat body tissues of the vectors. NCMV acquired by the vectors for 2 days, was recovered readily from the gut after 3 days, and minute amounts of virus were recovered from salivary glands, but not from the fat body. After 12 days, a high concentration of the virus was obtained from the gut, salivary glands and fat body. The recovery of the virus from gut and salivary glands in culture media after 10 days showed that NCMV can multiply in these excised organs of the vectors. The fact that no virus could be recovered from fat body tissues cultured in the media for 10 days, may suggest that the fat body tissues are not active sites of NCMV multiplication.

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