Studies on the Resistance of Cucumber to Cucumber Mosaic Virus II. Induction of Resistance by Infection

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Studies on the Resistance of Cucumber to Cucumber Mosaic Virus

II. Induction of Resistance by Infection

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Summary

The induction process of resistance in cucumber to cucumber mosaic virus (CMV) was studied using cotyledon disks. The time course of virus multiplication in the susceptible and the resistant cultivars was similar during the initial period of infection. The virus content in the resistant cultivar decreased after it reached a muximum (36 hr after inoculation), while the virus content in the susceptible cultivar remained at a constant level. When the cotyledons of the resistant cultivar were treated with actinomycin D (AMD, 10 μg/ml) at 6 or 12 hr after inoculation, virus multiplication was remarkably increased. However, a slight increase of virus multiplication in the susceptible cultivar was observed. The pattern of CMV multiplication in AMD-treated cotyledon disks of the resistant cultivar was similar to that in the untreated susceptible cultivar. Also the virus multiplication in the resistant cultivar was increased by α-amanitin and heat treatment.

It was concluded that the cells of the resistant cultivar have a capacity to support virus multiplication at the same level as those of the susceptible cultivar. But in the resistant cultivar the resistant is induced shortly after infection, so that the resistant cultivar allows only a low level of virus multiplication.

The interaction between CMV and cucumber cultivars which differed in susceptibility to infection has been studied using systemically infected leaves (1, 2, 3) or inoculated cotyledons (4). Resistance of cucumber to CMV is known to be controled by three or more genes (5, 6), but the mechanism of the resistance is not fully understood. In a previous paper (7), it was found that chlorotic lesions produced on CMV-inoculated cotyledons of a resistant cultivar (Shimoshirazu Zibai) did not enlarge after their appearance and remained much smaller than those of a susceptible cultivar (Best Green) which rapidly enlarged. The virus concentration in the cotyledons of the resistant cultivar was also much lower than that of the susceptible cultivar. It was assumed that virus multiplication or its movement is difficult in the cotyledons of the resistant cultivar by the induction of resistance.

Recently, it was reported (8, 9) that localization of tobacco mosaic virus and localized acquired resistance in hypersensitive hosts were partially inhibited by AMD which was known to inhibit DNA-dependent RNA synthesis in the cell (10). Partial suppression of resistance by the antibiotic was also reported in cucumber (11, 12). These reports support the possibility that a resistance-inducing substance is produced by the cellular transcription mechanism.

The studies reported here were undertaken to compare the time course of CMV multiplication in cotyledons of the susceptible and of the resistant cultivars in the presence or absence of AMD. In addition, the effects of α-amanitin, chloramphenicol and heat treatment on CMV multiplication were investigated, and the mechanism of resistance in cucumber cotyledons to CMV infection was discussed.

Materials and Methods

Plant: The two cucumber (*Cucumis sativus* L.) cultivars, Best Green (susceptible) and Shimoshirazu Zibai (resistant) were used. These plants were grown in a green house and the cotyledons were used about 10 days after sowing. For inoculation, the cotyledons were dusted with Carborundum and rubbed with a gause pad dipped into the inoculum.

Virus: The virus used in the experiments was the yellow strain of CMV. CMV was partially purified from the inoculated tobacco leaves (*Nicotiana tabacum* L., KY 57) according to Scott's method (13), stored at −20°C and diluted with 0.1 M phosphate buffer, pH 7.0 (1:20, v/v), before use.

Virus multiplication in the cotyledon disks: The cotyledons of each cultivar were inoculated with CMV solution (0.3 mg/ml in 0.005 M borate buffer, pH 9.0), at which concentration it causes complete collapse of the inoculated cowpea leaves, and produces numerous chlorotic lesions on the cucumber cotyledons of both cultivars. After inoculation the cotyledons were thoroughly washed with tap water, two disks (16 mm in diameter) per cotyledon were immediately excised with a cork borer. These disks were incubated on wet filter paper in a petri dish at 25–28°C to provide a photoperiod of 12 hr. At various intervals after inoculation, ten disks for each time period were harvested and stored frozen. The virus concentration in the cotyledon disks was determined as follows: the disks were thawed and ground with 5 ml of chloroform and 5 ml of 0.5 M citrate buffer, pH 6.5 (containing 0.1 per cent thioglycolic acid and 0.01 M EDTA). The homogenates were centrifuged at 7,800 ×g for 15 min and the supernatants were dialyzed against 0.005 M borate buffer, pH 9.0, for 20 hr. The dialyzed preparations were centrifuged at 7,800 ×g for 15 min, diluted with 0.1 M phosphate buffer, pH 7.0 (1:20, v/v), and were assayed for virus infectivity using local lesion method on cowpea primary leaves (*Vigna sinensis* Endl. *var. sesquipedalis*, cultivar Kurodane sanjaku). The local lesion number was counted after 24 hr of inoculation.

Application of antibiotics: The antibotics used in the experiments were actinomycin D (AMD, Boehringer Mannheim Co. Ltd., 10 μg/ml), α-amanitin (Boehringer Mannheim Co. Ltd., 40 μg/ml) and chloramphenicol (Sankyo Co. Ltd., 200 μg/ml). The cotyledons of each cultivar were inoculated with CMV and immediately washed with tap water. The disks were cut from the cotyledons at 6 hr after inoculation, and were floated on the solution containing antibiotics for 24 hr or on distilled water used as a control.

Heat treatment: The heat treatment was accomplished at 12 hr after inoculation by immersing the cotyledons in hot water (50±0.5°C) for 30 sec. The disks were then removed from each cotyledon and incubated on wet filter paper in a petri dish.

The virus concentration in the antibiotic- or heat-treated disks was determined 5 days after inoculation. These disks were washed, blotted dry, and ground with 0.1 M phosphate buffer, pH 7.0 (1:10, w/v). The extracts were assayed for virus infectivity on cowpea.

Results

Virus multiplication in cotyledon disks: Multiplication of CMV in cotyledon disks of susceptible and resistant cucumber cultivars was investigated for 96 hr after inoculation. The results of a typical experiment were shown in Fig. 1. The time course of CMV multiplication in both the susceptible and the resistant cultivars was similar during the initial period (0–36 hr) of infection. The virus increase

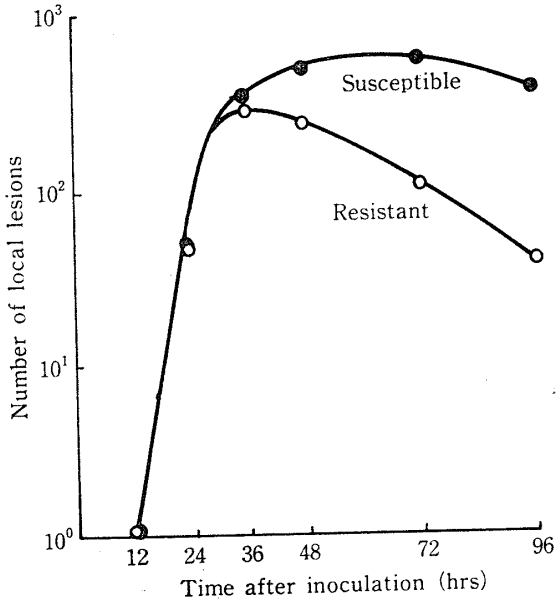

FIG. 1. Time course of CMV multiplication in cotyledon disks of the susceptible and the resistant cucumber cultivars. Virus was partially purified from each sample (10 disks of 16 mm diam.) and infectivity was assayed on cowpea primary leaves. Virus infectivity was represented as local lesion number per 10 cm^2 of leaf.

in the cotyledons of both cultivars started at 12 hr after inoculation and the virus content increased rapidly up to 36 hr. Thereafter the virus content in the resistant cultivar gradually decreased, whereas in the susceptible cultivar it remained nearly constant after 36 hr of infection. The virus concentration in the disks from the resistant cultivar was about 1/10 of that from the susceptible cultivar at 96 hr after inoculation. These results indicate that the virus multiplication was strongly suppressed after 36 hr in the cotyledons of the resistant cultivar, although during the early period of infection the virus was synthesized as actively in these tissues as in those of the susceptible cultivar.

Application of AMD at various times after inoculation: AMD was applied to the cotyledon disks at various times up to 96 hr after inoculation. The disks of each cultivar were floated on AMD solution for 24 hr, and then transferred on to filter paper moistened with distilled water in petri dishes. Fig. 2 shows that the effect of AMD on CMV multiplication is application time dependent. When AMD was applied to the cotyldeon disks of the resistant cultivar at 6 or 12 hr after inoculation, the virus concentration in the disks was increased to about eight times that in the untreated disks. It was difficult to distinguish whether the chlorotic lesions on the cotyledon disks of the resistant cultivar were enlarged or not by the treatment, because the disks were discolored by AMD treatment. The effect of AMD was progressively reduced when it was applied at periods later than 12 hr after inoculation. A little effect on the virus multiplication was observed when AMD was applied to cotyledon disks of the susceptible cultivar at 12 hr after inoculation, but the effect was not so remarkable as on the resistant cultivar.

CMV multiplication in the presence of AMD: The multiplication of CMV in cotyledon disks of the resistant cultivar in the presence of AMD was investigated. Disks were excised from CMV-inoculated cotyledons at 6 hr after inoculation and then floated on AMD solution for 24 hr. Thereafter, the disks were transferred on to filter paper wetted with distilled water in petri dishes. These disks were harvested at various intervals until 96 hr after inoculation. The results were shown in Fig. 3. Virus concentrations in AMD-treated and untreated disks began to rise from 12 hr of infection, and increased rapidly during the next 24 hr. The time course of CMV multiplication in the disks of both groups was similar up to 36–48 hr after inoculation. Thereafter, the virus content in AMD-treated disks remained a constant level, while that in the untreated disks gradually decreased. Thus the multiplication of CMV in AMD-treated disks of the resistant cultivar showed a similar pattern to that in the disks of the susceptible cultivar (Fig. 1). These results show that the induction of resistance which occurs in CMV-infected cucumber cotyledons was suppressed by the AMD treatment.

Effects of chloramphenicol and α-amanitin on CMV multiplication: It was reported that chloramphenicol is an inhibitor of protein synthesis by 70s

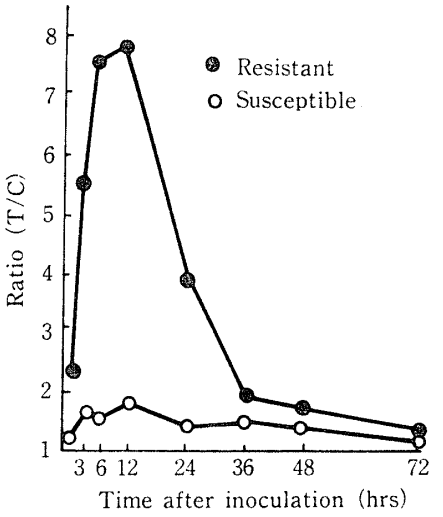

Fig. 2

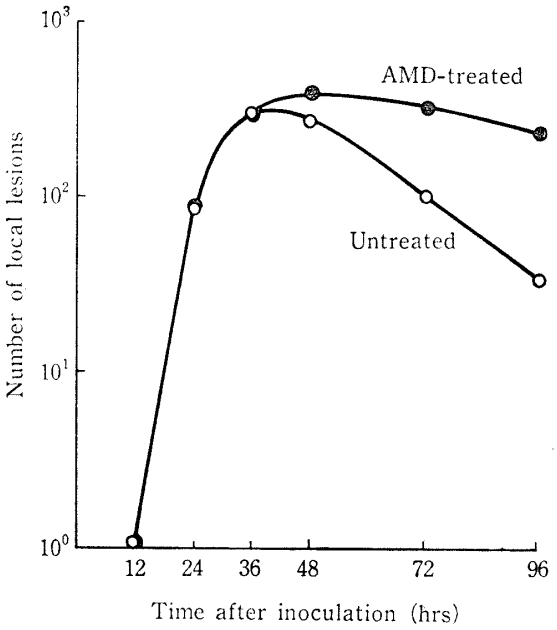

Fig. 3

FIG. 2. Effect of actinomycin D on CMV multiplication in cotyledons of the susceptible and the resistant cucumber cultivars. AMD was applied at different time intervals after inoculation. Virus infectivity was assayed 5 days after inoculation.
T: Infectivity in the extracts of AMD-treated cotyledons. C: Infectivity in the extracts of untreated cotyledons. The data represents the average of three experiments.

FIG. 3. Time course of CMV multiplication in cotyledon disks of the resistant cucumber cultivar in the presence of actinomycin D (10 μg/ml). Virus was partially purified from each sample (10 disks of 16 mm diam.) and infectivity was assayed on cowpea primary leaves. Virus infectivity was represented as local lesion number per 10 cm^2 of leaf.

ribosome (14) and that α-amanitin inhibits RNA-polymerase in the nucleus (15). As indicated in Table 1, these antibiotics were not effective on CMV multiplication in the susceptible cultivar. On the other hand, virus multiplication in the resistant cultivar was remarkably increased when the cotyledons were treated with α-amanitin. Chloramphenicol had little effect on CMV multiplication at the concentration used, which was reported (16) to stimulate the multiplication of tobacco mosaic virus in cucumber cotyledons when it was applied within 1 day of infection.

Effect of heat treatment on CMV multiplication: Yarwood (17) found that brief heat treatment of bean primary leaves after tobacco mosaic virus inoculation increased the infectivity of leaf extracts as well as the lesion size. Similar results were reported by Wu *et al.* (18).

Therefore, this experiment was conducted to determine if the virus production in the cucumber cotyldeons increases with heat treatment. Table 2 shows the effect of heat treatment on CMV multiplication in the cotyledons. The virus content in the heat treated cotyledons of the resistant cultivar increased about

TABLE 1. *Effects of Chloramphenicol and α-Amanitin on CMV Multiplication*

	Number of local lesions[a]					
	Susceptible		T/C	Resistant		T/C
	C	T		C	T	
Chloramphenicol (200 μg/ml)	561 887	600 754	1.07 0.85	48 72	69 92	1.44 1.28
α-Amanitin (40 μg/ml)	798 629	843 646	1.06 1.01	125 81	422 355	3.38 4.38

C: Untreated T: AMD-treated

a: For each assay 10 leaves were used. Number of local lesions produced by the extracts of cotyledon disks was represented per 10 cm^2 of leaf.

TABLE 2. *Effect of heat treatment on CMV multiplciation*

	Number of local lesions[a]		
	Untreated (C)	Heat-treated (T)[b]	T/C
Susceptible	625 861	604 880	0.97 1.02
Resistant	84 70	501 428	5.96 6.07

a: For each assay 10 leaves were used. Number of local lesions produced by the extracts of cotyledon disks was represented per 10 cm^2 of leaf.

b: Cotyledons were immersed in hot water (50°C) for 30 sec. 6 hr after inoculation.

four-fold over that of the untreated cotyledons. However, there was no effect on CMV multiplication in the susceptible cultivar. These results suggest that brief heat treatment shortly after inoculation reduces the resistance in cotyledons of the resistant cultivar.

Discussion

Following CMV inoculation on cotyledons of the susceptible and the resistant cucumber cultivars, chlorotic lesions are produced. As described in the previous report (7), these lesions on the resistant cultivar did not enlarge, while those on the susceptible cultivar enlarged gradually. The time course of CMV multiplication in both cotyledon disks of the resistant and the susceptible cultivars was very similar for 36 hr after inoculation. Thereafter, virus multiplication in the resistant cultivar slowed down, so that the final virus concentration attained was much lower than that of the susceptible cultivar. These results suggest that the virus multiplication in the resistant cultivar is similarly active as that in the susceptible cultivar at least for 36 hr after inoculation. When AMD was applied

at 6 or 12 hr after inoculation, CMV multiplication in the resistant cultivar was significantly increased. The time course of CMV multiplication in AMD-treated cotyledon disks showed a similar pattern as that in untreated disks from the susceptible cultivar. Recently, enhancement of CMV multiplication in cucumber cotyledons by application of AMD was reported by Nachman *et al.* (11) and Barbara and Wood (12) with different resistant cucumber cultivars. They have postulated that AMD might inhibit the induction of resistance in the cotyledons. It was observed in addition to the effect of AMD that treatments of heat and α-amanitin also stimulated the virus multiplication in cotyledons of the resistant cultivar. α-Amanitin inhibits the RNA-polymerases located in the nucleoplasm (15). These facts suggest that the resistance may be induced by a substance or substances which are produced during an early stage of infection through active processes involving cellular transcription system. Moreover, heat treatment enhanced the virus multiplication in cotyledons of the resistant cultivar. Although the nature of the heat-sensitive factors in the cotyledons is not known, it is assumed that it affects some proteins (19).

On the basis of these findings, it was considered that the cotyledon cells of the resistant cultivar support active CMV synthesis at the same level as those of the susceptible cultivar before the resistance is induced. Though it is not clear whether the restriction of virus movement is involved or not in this resistant mechanism, it is possible that the new virus synthesis in infected cells is suppressed after induction of resistance, in which mechanism a substance(s) produced by DNA-RNA system may be involved.

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