タバコモザイクウイルス感染タバコおよびグルチノーザ葉内のウイルスの増殖および核酸合成に対するシトリニンの影響

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Effect of Citrinin on TMV Multiplication and RNA Synthesis in Infected Tobacco and *Nicotiana glutinosa* Leaves

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安田　康*・片岡正明*・細辻豊二**・野口照久*：タバコモザイクウイルス感染タバコおよびグルチノーザ葉内のウイルス増殖および核酸合成に対するシトリニンの影響

Abstract

Citrinin, which markedly inhibits local lesion formation on *Nicotiana glutinosa* leaves infected with tobacco mosaic virus (TMV), inhibited TMV multiplication in tobacco and *N. glutinosa* leaves. TMV multiplication was inhibited by 70% when leaf-discs from inoculated leaves were floated on 100 ppm citrinin solution for 48 hours. The inhibition rate increased progressively with the time of treatment, while TMV produced in treated leaves had the same specific infectivity as that produced in untreated leaves. When ^{14}C-amino acid was infiltrated into infected *N. glutinosa* leaves and labeled TMV was separated by gel filtration, citrinin was found to inhibit the incorporation of ^{14}C into TMV. Effect of citrinin on RNA synthesis in tobacco and *N. glutinosa* leaves infected with TMV was investigated by using methylated albumin kieselguhr (MAK) column chromatography and disc electrophoresis in polyacrylamide gel. In the MAK column chromatographic pattern, TMV-RNA overlaps with 28S rRNA peak. Citrinin inhibited the incorporation of ^{3}H-uridine into 28S rRNA fraction of both uninfected and infected leaves, whereas the incorporation into sRNA, DNA-like RNA, and 18S rRNA was inhibited only to a negligible extent. Analyses using the polyacrylamide gel electrophoresis showed that citrinin inhibited cell RNA synthesis to some extent even at 25 ppm. Citrinin, at a low concentration, inhibits the 28S rRNA but at a high concentration, 100 ppm, inhibits both 28S rRNA and TMV-RNA synthesis. Since citrinin at 75 ppm completely inhibits local lesion formation on *N. glutinosa* while inhibits TMV synthesis by about 50%, the effect of citrinin on lesion formation is not directly connected with that on virus synthesis but the effect may be caused via the suppressed function of ribosomes.

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Introduction

Citrinin, which was isolated from cultures of *Penicillium* sp. AK-019, was reported to have a markedly inhibitory effect on local lesion formation on *Nicotiana glutinosa* leaves caused by tobacco mosaic virus (TMV)[21]. As one of the inhibition mechanism, inhibition of peroxidase and polyphenoloxidase activities in *N. glutinosa* leaves was investigated[22]. In general, local necrosis in infected *N.*

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glutinosa leaves occurs only after virus has been synthesized[13,17,21,23]. Therefore, it may be reasonable to assume that citrinin reduces the rate of TMV multiplication in infected leaves, thus inhibiting local lesion formation on *N. glutinosa* leaves.

Virus biosynthesis may influence the synthesis of normal cell RNA to a considerable extent[1,12,14,15]. Effect of citrinin on RNA synthesis in virus-infected leaves seems to be an important problem for solving the question whether it acts directly on viral RNA synthesis or not. This paper describes the effect of citrinin on TMV multiplication and RNA synthesis in tobacco and *N. glutinosa* leaves.

Materials and methods

Virus, plants and inoculation Upper surfaces of detached and matured tobacco (*Nicotiana tabacum* L., Bright Yellow) and *Nicotiana glutinosa* leaves were dusted with carborundum, inoculated with 0.1–0.5 mg/m*l* of purified TMV (ordinary strain), and immediately washed with running tap water.

Citrinin treatment Ten mg of citrinin was dissolved in 1.0 m*l* of acetone and diluted to a given concentration with distilled water. Leaf-discs, 20 mm in diameter, were punched with a cork borer from inoculated leaves. They were floated on citrinin solutions or distilled water in petri dishes and incubated at 25°C under continuous illumination (2,000 lux) from fluorescent lamps.

Isotopes treatment At given hours from inoculation, tobacco or *N. glutinosa* leaf-discs were transferred to ^{3}H-uridine (5–12.5 μCi/m*l*) or ^{14}C-uridine (2.5 μCi/m*l*) that still contained citrinin, and incubated for the last given hours during the incubation of 48 hours. ^{14}C-glycine (1.0 μCi/m*l*) was infiltrated through the petioles into *N. glutinosa* leaves.

Separation of ^{14}C-labeled TMV by agar-gel filtration ^{14}C-labeled TMV in *N. glutinosa* leaves was separated by the method of agar-gel filtration[19]. A glass tube for chromatography was packed with 4% agar chips having a 30–60 mesh. The column (1.5 × 100 cm) was washed with sufficient 0.2 M sodium phosphate buffer (pH 7.0). The infected and isotope-treated 30–50 fresh leaves were homogenized with 75 m*l* of sodium phosphate buffer. After added 5 m*l* of the purified 'cold' TMV (2.5 mg/m*l*) as a carrier, virus nucleoprotein was precipitated by adding ammonium sulfate and adjusting the pH to isoelectric point of the virus (pH 3.4). The pellet was dissolved in the same buffer and layered on the top of the column. TMV fraction was eluted by the same buffer at room temperature. Each 4 m*l* of the effluent was assayed for its ultraviolet absorption at 260 mμ using a spectrophotometer (Hitachi 101 type). From each fraction, 0.5 m*l* was put into a counting vial and 10 m*l* of Bray's scintillator[3] was added. The radioactivity was determined by using a liquid scintillation spectrometer (Packard Tri-Carb 3320).

Nucleic acids extraction Nucleic acids from leaf-discs were extracted by phenol by the method of Schlegel[16] and modified by Kubo *et al.*[11] After treated with isotope, 70–80 leaf-discs were homogenized by grinding with mortar and pestle in 20–30 m*l* of a mixture of 1% tetrasodium pyrophosphate and water-saturated phenol (1:1), and then centrifuged. The aqueous phase was allowed to stand overnight by adding 3–4 times their volumes of alcohol and the pellet was collected, finally dissolving in 10 m*l* of 0.4 M NaCl-0.05 M phosphate buffer (pH 6.7). Before charging on MAK column or layering on polyacrylamide gel, it was dialyzed for 10 hours. All operations of nucleic acids extraction were done in a cold room.

Fractionation of nucleic acids on methylated albumin kieselguhr (MAK) column The MAK column was prepared by the method of Kubo *et al.*[11] as follows: 20 g of Hyflosupercel were suspended in 80 m*l* of 0.4 M NaCl-0.05 M phosphate buffer (0.4 M PBS), pH 6.7, and mixed with

7 m*l* of 1% methylated bovine serum albumin. This mixture was poured into a glass tube to make a column (2×16 cm) having a thin layer which was formed at the top by mixing 1 g Hyflosupercel and 10 m*l* of 0.4 M PBS. The charged nucleic acid was eluted at room temperature with 500 m*l* of NaCl-phosphate buffer (pH 6.7) by a linear gradient concentration from 0.4 M to 1.4 M. The optical density at 260 mμ of each 4 m*l* of the effluent was measured. From each fraction, 0.5 m*l* was put into a counting vial, adding the scintillator and the radioactivity was determined as described above.

Gel electrophoresis of nucleic acids Polyacrylamide gel electrophoresis was carried out by the modified procedure of Bishop *et al.*[2] and that of Hirai and Wildman[7] by using disc electrophoresis apparatus (Mitsumi SJ-1050B type). The gels were composed of 1.8 m*l* of 15% acrylamide and 0.75% methylene-bis (acrylamide), 1.0 m*l* of glycerol, 5.0 m*l* of '2E-buffer' (0.08M Tris-0.04 M sodium acetate-0.002 M EDTA, adjusted the pH to 7.2 with acetic acid), and 2.1 m*l* of water· After 0.08 m*l* of 10% ammonium persulfate and 0.01 m*l* of *N,N,N',N'*-tetra-methyl-ethylene-diamine were added as the catalyst system, the mixture was allowed to polymerize in 6 mm diameter and 70 mm long glass tubes. Amount of 50–100 μg of nucleic acid in 40 μl of 'E-buffer' (half concentration of '2E-buffer') containing 20% glycerol was then layered over the gels and the electrophoresis was run for 90–120 minutes at room temperature. After that, the gels were stained overnight in acetate buffer, pH 4.7, with 0.2% methylene blue and washed with running tap water. For ^{14}C counting, the gels were frozen by means of dry ice, sliced into 1 mm sections, and were hydrolyzed overnight by 1.0 m*l* of hydrogen peroxide at 70°C. Ten m*l* of scintillator was added and the radioactivity was determined.

Chemicals Citrinin was purified by finally adding ethanol after isolated from cultures of *Penicillium* sp. AK-019[21,22]. ^{3}H-uridine (specific activity 5 Ci/mM) and ^{14}C-uridine (specific activity 60 mCi/mM) were purchased from the Radiochemical Centre, Amersham, England. ^{14}C-glycine (specific activity 4.0 mCi/mM) was purchased from the Daiichi Pure Chemicals Co., Ltd. Tokyo.

Results

Inhibition by citrinin of TMV multiplication in tobacco and *N. glutinosa* leaves

In preliminary experiments, TMV multiplication was progressively inhibited with the prolonged times of 100 ppm citrinin-treatment. Fig. 1 shows the decreases in TMV synthesis in tobacco leaf-discs treated with 100 ppm citrinin and the time course inhibition by this substance. In this case,

Table 1. Effect of citrinin on the infectivity of TMV synthesized in tobacco leaves

Hours of treatment	$OD_{260m\mu}$ (X)	No. of lesions per half leaf (Y)	Specific infectivity (Y/X)
0	1.55	216	139
5	0.84	124	147
24	0.48	67	137
48	0.37	47	138
72	0.21	30	141

After the amount of TMV in the inoculated leaf-discs, which were floated on the solution of 100 ppm citrinin for given hours, was determined by the method of Taniguchi[20], the number of local lesions produced by inoculating *N. glutinosa* leaves with TMV was counted.

the rate of inhibition showed the same trend in four independent experiments, and TMV synthesis was inhibited by 70% by the treatment for 48 hours. Number of local lesions produced per unit concentration of the virus, which was synthesized in tobacco leaf-discs treated with 100 ppm citrinin, was compared among various hours of treatment. Table 1 shows that the specific infectivity of TMV in tobacco leaves never decreased with the prolonged times of treatment. Thus, citrinin produced TMV having the normal infectivity, although the rate of synthesis decreased by the treatment.

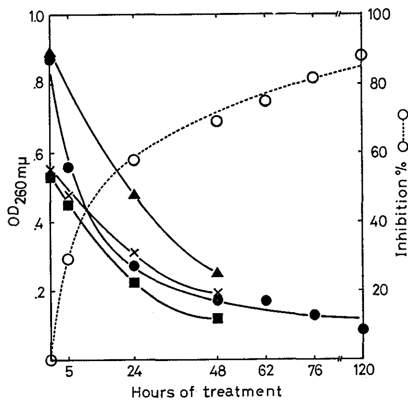

Fig. 1. Decreases in TMV synthesis in tobacco leaf-discs treated with 100 ppm citrinin and the time course inhibition.

The amount of TMV was determined by Taniguchi's method[20]. Triangles: experiment-1, squares: -2, filled circles: -3, crosses: -4, open circles: percent of inhibition.

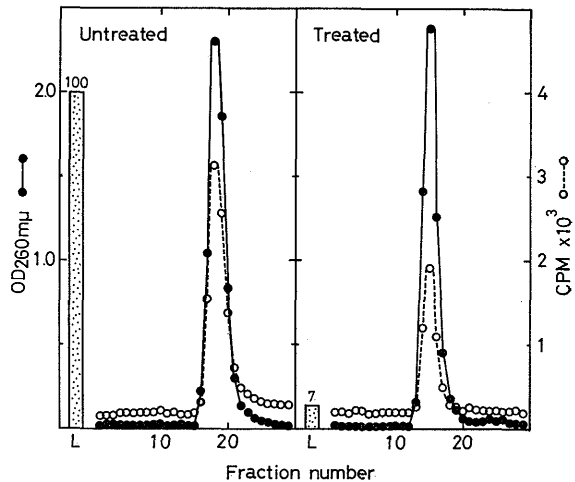

Fig. 2. Incorporation of ^{14}C-glycine into TMV synthesized in *N. glutinosa* leaves treated with citrinin and the opticaldensity of TMV which contains the virus added as a marker.

Separation by agar-gel filtration. ^{14}C-glycine and 75 ppm of citrinin were infiltrated into leaves through the petiole for 48 hours immediately after inoculation. Filled circles: optical density at 260 mμ, open circles: ^{14}C-radioactivity, L: relative number of local lesions.

As have been reported[21], citrinin markedly inhibited local lesion formation on *N. glutinosa* leaves caused by TMV and the rate of inhibition of TMV synthesis in the same leaves increased with lprolonged times of treatment and the increased concentration of citrinin, in the same way as in tobacco eaves. This inhibitory effect of citrinin was confirmed in Fig. 2 which shows that, when ^{14}C-glycine was incorporated into TMV that is synthesizing in *N. glutinosa* leaves treated with 75 ppm citrinin, the amount of TMV, labeled during 48 hours after inoculation, decreased by about 50% of the untreatment. The inhibitory effect of citrinin on the local lesion formation far exceed that on the virus synthesis. This is consistent with the results in our previous report[21].

Effect of citrinin on RNA synthesis in tobacco and *N. glutinosa* leaf-discs infected with TMV

To separate nucleic acid fractions produced in leaf-discs, MAK column was used. Each of two chromatographic patterns, shown in Fig. 3, of nucleic acids from tobacco leaves 48 hours after inoculation with TMV and from healthy tobacco leaves had four main peaks which corresponded to, in their order of elution, soluble RNA (sRNA), DNA, 18S and 28S ribosomal RNA (rRNA), according

to Kubo *et al.*[11] and Itho and Hirai[10]. The radioactivity pattern of host-RNA's was almost parallel with the UV-absorbing pattern except for the DNA-like RNA and the radioactivity was the highest at 28S rRNA, when labeled for 6 hours at 42 hours after inoculation. The rate of incorporation of ^{3}H-uridine into the infected-leaf RNA's was similar to that into uninfected samples, and no characteristic differences in their chromatographic patterns were observed between infected and uninfected leaf-discs. The incorporation of ^{3}H into TMV-RNA synthesized, however, was very often detected as a small shoulder, although TMV-RNA could not be completely separated from host-rRNA (28S).

Experiments were performed to confirm whether or not citrinin inhibits RNA synthesis in tobacco leaves. The uninfected and infected leaf-discs punched from half-leaves were floated on 75 ppm citrinin for 48 hours after inoculation and the leaf-discs from other halves on water for the same times. ^{3}H-uridine was applied for the last 6 hours during the incubation. The radioactivity of the uninfected-leaf RNA's was the highest at 28S rRNA (Fig. 4a). In citrinin-treated samples, the rate of incorporation of ^{3}H into the 28S rRNA decreased below that of the untreated samples, suggesting that citrinin inhibited 28S rRNA synthesis to some extent (Fig. 4b). The radioactivity pattern of the infected-leaf RNA's showed a similar trend to that of uninfected tissues with the exception of having a small TMV-RNA peak (Fig. 4c). In citrinin-treated and infected leaf-discs, the incorporation of ^{3}H-uridine into the 28S rRNA was suppressed in the same way as in uninfected ones, suggesting that both 28S rRNA and TMV-RNA syntheses were inhibited by citrinin-treatment (Fig. 4d). The specific radioactivity (counts/optical density) of 28S was lowered as compared with that of 18S rRNA in treated leaf-discs, in contrast with untreated samples, in which the incorporation rate into 28S was higher than that into 18S rRNA. Much more ^{3}H was incorporated into both peaks of sRNA

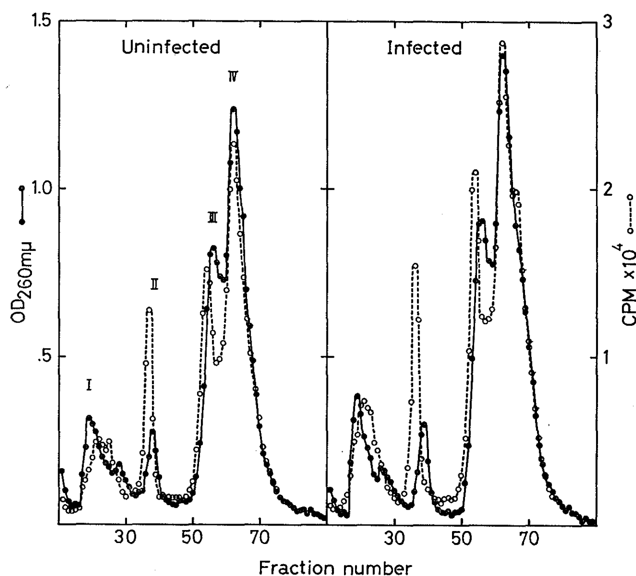

Fig. 3. Fractionation of nucleic acids extracted from uninfected and from TMV-infected tobacco leaf-discs 48 hours after inoculation, with MAK column chromatography.

^{3}H-uridine was applied to leaf-discs for the last 6 hours during the incubation of 48 hours. Filled circles: optical density at 260 mμ, open circles: ^{3}H-radioactivity, peak I: sRNA, peak II: DNA, peak III: 18S rRNA, peak IV: 28S rRNA.

and DNA-like RNA in citrinin-treated than into those in untreated leaves.

The same experiment was performed with *N. glutinosa* leaves. ^{3}H-uridine was applied for the last 18 hours during the 48 hour incubation period. The radioactivity of infected and uninfected-leaf RNA's was the highest at 28S rRNA (Fig. 5a, b). Local lesions formed on *N. glutinosa* leaves caused by TMV appeared about 36 hours after inoculation. During 30–48 hours after inoculation, the rate

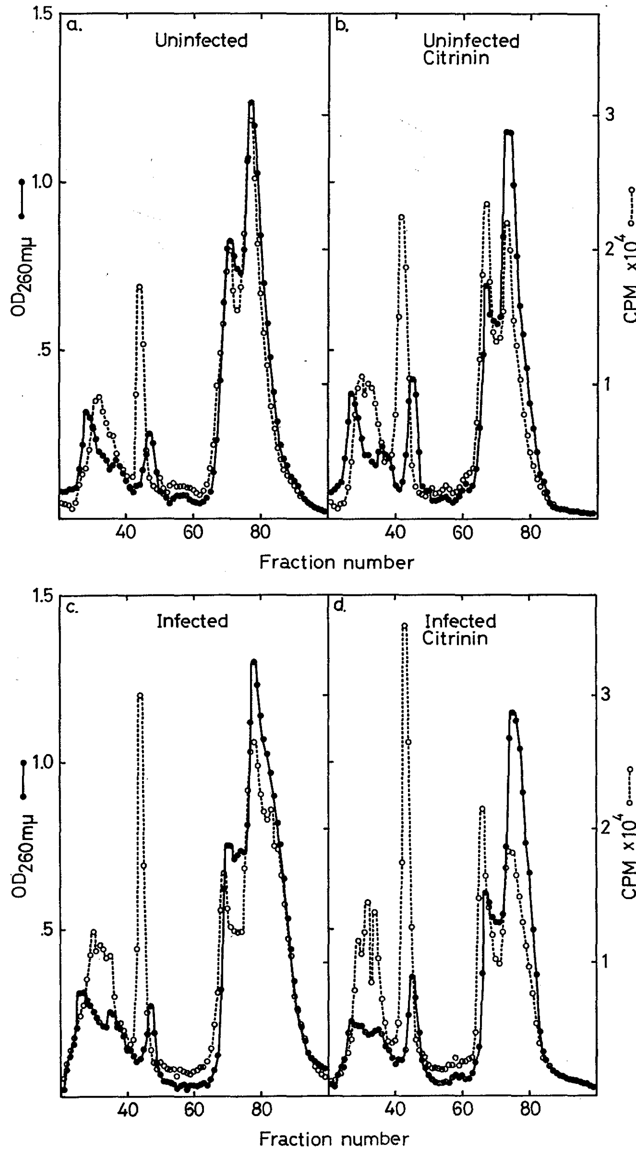

Fig. 4. Fractionation of nucleic acids of uninfected and TMV-infected tobacco leaf-discs treated with citrinin, with MAK column chromatography.

^{3}H-uridine was applied to leaf-discs, treated with 75 ppm of citrinin, for the last 6 hours during the incubation of 48 hours. (a) Uninfected control. (b) Uninfected and citrinin-treated. (c) Infected control. (d) Infected and citrinin-treated. Symbols: see Fig. 3.

of incorporation of ^{3}H-uridine into 28S rRNA fraction which contains TMV-RNA considerably increased, while no changes in the syntheses of the other nucleic acids were observed in infected leaf-discs. This suggests that rRNA and viral RNA syntheses increased during the infection stages. In the case of the almost complete inhibition of lesion formation by treatment with 75 ppm citrinin, the incorporation of ^{3}H-uridine into 28S rRNA was markedly suppressed (Fig. 5c).

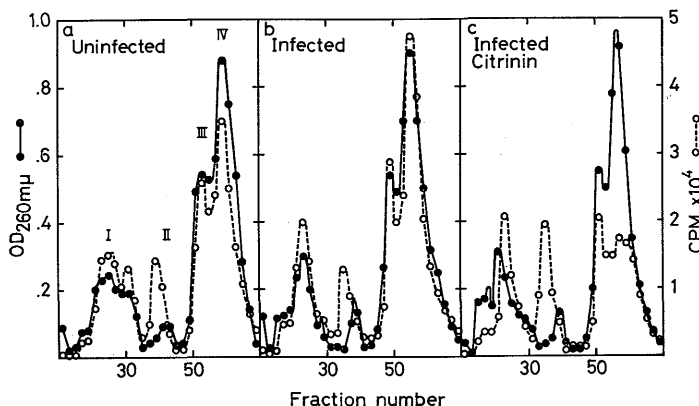

Fig. 5. Fractionation of nucleic acids of TMV-infected leaf-discs of *N. glutinosa* treated with citrinin, with MAK column chromatography.

^{3}H-uridine was applied to leaf-discs, treated with 75 ppm of citrinin, for the last 18 hours during the incubation of 48 hours. (a) Uninfected. (b) Infected. (c) Infected and citrinin-treated. Symbols: see Fig. 3.

Effect of citrinin on the host- and virus-RNA syntheses in tobacco leaf-discs

Fig. 6 shows diagramatically the gel electrophoresis patterns of the nucleic acids extracted from tobacco leaf-discs and TMV-RNA. Five and 6 bands in uninfected and TMV-infected leaves, respectively, were detected in the stained materials. From the position of purified TMV-RNA (Fig. 6e), TMV-RNA (band II) could be clearly demonstrated and separated from host RNA's (band III–VI) which contain 28S and 18S rRNA's, according to Hirai and Wildman[7]. No difference in their electrophoretic patterns were observed between untreated and treated leaves when incubated for 48 hours with 25 ppm citrinin. Band I is probably an unknown contaminant contained in RNA extraction.

Fig. 7 shows the electrophoresis pattern of ^{14}C-labeled nucleic acids extracted from uninfected and from TMV-infected tobacco leaf-discs 48 hours after inoculation. The radioactivity of nucleic acid components almost corresponded with the positions of stained bands as shown in Fig. 6 with the exception of band V and VI, when labeled for 24 hours from inoculation. The radioactivity was the highest at band III in Fig. 6, which was presumably 28S rRNA in uninfected and infected leaves (peak III). In addition, radioactive TMV-RNA was separated from host-RNA's. The incorporation of ^{14}C-uridine into band V and VI components was not clearly recognized in this experiment.

In citrinin-treated samples, the rate of incorporation of ^{14}C-uridine into total RNA's was reduced as compared with that in untreated samples, as shown in Fig. 8, suggesting that citrinin suppressed RNA synthesis in tobacco leaves when treated for 48 hours. The rate of inhibition of peak III-RNA synthesis in uninfected leaves was higher than that in infected leaves. In this case, TMV-RNA synthesis was slightly inhibited, but citrinin, even at 25 ppm, inhibited much more syntheses of host-RNA's than that of TMV-RNA.

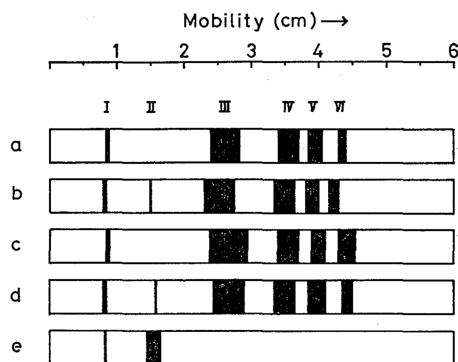

Fig. 6. Diagramatic representation of gel electrophoresis of nucleic acids extracted from uninfected and TMV-infected tobacco leaf discs 48 hours after inoculation and TMV-RNA.

Amount of 56.6 μg of nucleic acids was layered on the 2.7% gels and an electrophoresis was run for 90 minutes at 5 mA per tube. (a) Uninfected. (b) Infected. (c) Uninfected and citrinin (25 ppm)-treated. (d) Infected and citrinin (25 ppm)-treated. (e) TMV-RNA, which was extracted from purified virus by the phenol method[5]. Band I: unknown contaminant, band II: TMV-RNA, bands III-VI: host-RNA's.

Discussion

Citrinin was found to inhibit TMV multiplication in tobacco and *N. glutinosa* leaves. The inhibition in tobacco leaves reached to about 70% at 100 ppm. Its inhibitory effect increased with the prolonged times of treatment in contrast with that of Blasticidin S, which inhibits TMV multiplication and is effective by applying at early infection stages[8]. In the case of *N. glutinosa*, citrinin inhibited lesion formation more intensely than the virus synthesis (Fig. 2). Therefore, the hypothesis that citrinin inhibits TMV multiplication, thus inhibiting local lesion formation, could not be clearly confirmed in the present experiment. This may suggest, however, that the reduction of virus synthetic rate directly causes inhibition of local lesion formation but the reverse is not always true.

In order to confirm how extensively citrinin acts on RNA synthesis in infected leaves, analysis by means of MAK column chromatography was performed. The chromatographic patterns revealed that citrinin specifically inhibited 28S rRNA synthesis which involves TMV-RNA, whereas the syntheses of sRNA, DNA-like RNA[9], and 18S rRNA were not suppressed. It was also shown that the specific radioactivity of 28S was lowered as compared with that of 18S rRNA in citrinin-treated leaves, in contrast with untreated leaves. These facts indicate that citrinin inhibits the syntheses of both 28S rRNA and TMV-RNA. The question arises whether or not citrinin acts directly on the virus RNA or on host RNA metabolism. To obtain more effective separation of host- and TMV-RNA's, polyacrylamide gel electrophoresis was employed. TMV-RNA could be clearly separated from host RNA's. Citrinin affected the syntheses of host RNA's which includes 28S rRNA even at low concentration, although TMV-RNA synthesis was scarcely inhibited. Thus, it can be concluded that citrinin may suppress the syntheses of both 28S rRNA and TMV-RNA, thus inhibiting TMV multiplication at a high concentration and that it may not be a specific inhibitor against TMV-RNA

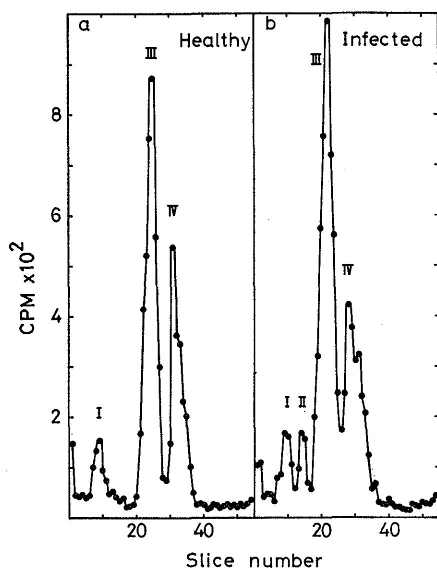

Fig. 7. Gel electrophoresis of ^{14}C-labeled nucleic acids extracted from uninfected and from TMV-infected tobacco leaf-discs at 48 hours after inoculation.

^{14}C-uridine was applied to leaf-discs for the last 24 hours during the incubation of 48 hours. After electrophoresis under the same conditions as in Fig. 6, the gel was sliced into 1 mm sections which were hydrolyzed by H_2O_2 and the radioactivity was counted. (a) Uninfected. (b) Infected. Symbols of peaks correspond to the respective bands in Fig. 6.

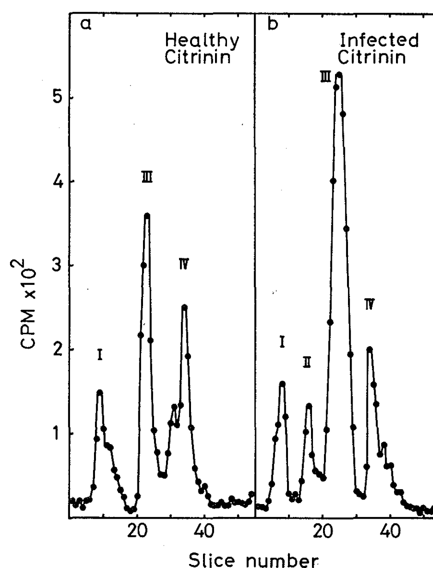

Fig. 8. Gel electrophoresis of ^{14}C-labeled nucleic acids extracted from uninfected and from TMV-infected tobacco leaf-discs, treated with 25 ppm of citrinin.

Conditions were the same as in Fig. 7 with the exception of treatment with citrinin. (a) Uninfected. (b) Infected. Symbols: see Fig. 7.

at a low concentration.

It was reported that host-RNA synthesis induced by virus infection increased at early stages of infection[1,12,15] and then decreased 48 hours after inoculation, while the relative rate of TMV-RNA synthesis was the highest at this stage[12]. In spite of the difference in experimental condition, a similar trend was observed in Figs. 3 and 5. It is remarkable that the synthesis of rRNA in infected *N. glutinosa* leaves was stimulated as compared with that in uninfected leaves, although it was not detected whether the increase in the synthesis actually occurred before or after lesion appearance. When lesion formation on *N. glutinosa* leaves was completely inhibited by citrinin-treatment, synthesis of 28S rRNA was considerably suppressed as the similar way as shown in tobacco leaves (Fig. 5), which suggests that there may be an interaction of lesion formation and synthesis of rRNA or ribosome. In order to find the inhibitory machanism by citrinin of local lesion formation caused by virus infection, further studies are needed to determine the effects of citrinin on host metabolism, especially, on protein synthesis including newly formed oxidative enzymes[4,18] and protein(s) which are necessary for virus-RNA biosynthesis[6].

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和文摘要

タバコモザイクウイルス感染タバコおよびグルチノーザ葉内のウイルスの増殖および核酸合成に対するシトリニンの影響

安田　康・片岡正明・細辻豊二・野口照久

タバコモザイクウイルス (TMV) 感染による局部病斑形成を顕著に阻止するシトリニンによって，タバコおよびグルチノーザ葉内のウイルスの増殖が阻害された。シトリニンの 100 ppm 溶液にタバコ葉ディスクを48時間浮かべた場合，TMV の増殖量は 30% まで減少した。処理葉内の TMV の増殖率は処理時間を長くするにしたがって低下したが，同葉内で増殖したウイルスの感染性は無処理葉内のそれと変わらなかった。またグルチノーザ葉内の TMV の増殖率を ^{14}C- アミノ酸のとりこみでしらべた場合でも，同様にシトリニン処理によって，ウイルスの増殖の阻害が認められた。

シトリニンの感染葉における核酸合成に対する影響を MAK カラムクロマトグラフィーおよびディスク電気泳動法によって検討した。MAK カラムクロマトグラフィーのパターンから，シトリニンは感染タバコおよびグルチノーザ葉の sRNA, DNA-likeRNA, および 18S rRNA のいずれの合成をも阻害しないが，TMV-RNA を含む 28S rRNA の分画への標識ウリジンのとりこみを抑制することが示された。またディスク電気泳動法による分析の結果，シトリニンを低濃度で処理した場合でも，タバコ葉の RNA 合成は幾分阻害された。シトリニンは低濃度でも 28S rRNA の合成を抑制するが，高濃度では 28S rRNA および TMV- RNA の両合成を阻害することが結論された。75 ppm のシトリニンはグルチノーザ葉上の局部病斑形成を完全に阻止するが，ウイルスの増殖を約 50% しかおさえなかった。これらのことから，シトリニンの病斑形成に対する阻止効果はウイルスの増殖に対する効果よりも，むしろリボゾームの機能を抑制することと関係があるものと推定された。