Isolation of TMV Synthesized in Infected Nicotiana glutinosa Leaves Treated with Citrinin by Using Agar-Gel Filtration

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Abstract

For separating 14C-labeled tobacco mosaic virus (TMV) from infected Nicotiana glutinosa leaves exposed with 14CO₂, agar-gel filtration was successfully utilized. The 14CO₂ fixed through photosynthesis effectively incorporated into TMV in the infected leaves and the radioactive virus was completely separated from leaf components by this method with the column packed with 4% agar chips having a 30-60 mesh.

In the 14CO₂-exposed N. glutinosa leaves treated with 25ppm citrinin for 48 hours immediately after inoculation, the number of local lesion caused by TMV was 1.4% of that of untreated leaves and the radioactivity of 14C-labeled TMV separated by similar treated leaves decreased by about 20% of the untreated. Citrinin may act on virus synthesis in N. glutinosa leaves at early infection stages, whereas it inhibits local lesion production even when treated 24 hours after the inoculation in the incubation chamber. (Received August 14, 1973)

Introduction

Steere and Ackers⁴ described the use of columns of granulated agar-gel for purification and separation of viruses. We have attempted to apply the similar columns for tobacco mosaic virus (TMV) separation from infected Nicotiana glutinosa leaves, in which the rate of virus multiplication is reasonably far less than that in Nicotiana tabacum var. Bright Yellow, a systemic host for TMV. Since 14CO₂ fixed through photosynthesis can effectively incorporate into TMV in tobacco leaves³,⁹, this method was utilized to estimate the rate of TMV multiplication in N. glutinosa leaves.

Citrinin (Fig. 1) has a remarkable inhibitory effect on local lesion formation on N. glutinosa leaves caused by TMV⁷. The inhibition mechanism, including the inhibition of oxidases activities⁶, of virus multiplication and of ribosomal RNA synthesis⁶ in tobacco and N. glutinosa leaves was reported.

This paper describes the separation of 14C-labeled TMV from 14CO₂-exposed N. glutinosa leaves and the effect of citrinin on TMV multiplication in N. glutinosa leaves by using agar-gel filtration.

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Fig. 1. Chemical structure of citrinin, 4, 6-dihydro-8-hydroxy-3, 4, 5-trimethyl-6-oxo-3H-2-benzopyran-7-carboxylic acid.
Materials and methods

Virus, plants and inoculation Upper surfaces of detached mature tobacco (N. tabacum L. var. Bright Yellow) and N. glutinosa leaves were dusted with carborundum, inoculated with 0.1-0.5 mg/ml of purified TMV (ordinary strain), and immediately washed with running tap water.

Preparation of 14C-labeled TMV Eighty tobacco leaf-discs, 20 mm in diameter, were punched with a cork borer from inoculated leaves. After floated on distilled water in a petri dish for 48 hours, they were transferred to 14C-leucine (46 μCi/15 ml/petri dish) and incubated for 48 hours at 25°C under continuous illumination (ca 2,000 lux) from fluorescent lamps. 14C-labeled TMV was purified by Taniguchi's method6). From the determination of optical density at 260 nm and the radioactivity of 14C-labeled TMV after dialyzed overnight against water, specific radioactivity of 14C-labeled TMV was calculated as 35.3×10⁴ cpm/mg.

Incorporation of 14CO₂ into infected N. glutinosa leaves Petioles of inoculated leaves of N. glutinosa were put in water or citrinin solution in a petri dish, which were placed in an acrylic acid resin chamber (50×50×10 cm). An apparatus producing 14CO₂ was connected to the chamber, in which air-pressure was reduced to about half atmospheric one. Air without CO₂, passed through a trap of 2N NaOH solution, adding 14CO₂ which was produced by adding 1N HCl to the mixture of Ba14CO₃ (4-18 mg) and BaCO₃ as a carrier, was filled in a hermetically sealed chamber. Leaves in the chamber were exposed to 14CO₂ (ca 0.3% CO₂, 0.02-0.08 μCi/ml air) for 24 or 48 hours in light (ca 10,000 lux) at 25°C. The air in the chamber was constantly stirred by a fan.

Separation of 14C-labeled TMV by agar-gel filtration Agar-gel filtration was carried out by the modified procedure of Steere and Ackers4), as have been reported6). 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). TMV from infected N. glutinosa leaves was prepared by the modified Taniguchi's method5). The infected and isotope-treated fresh leaves were homogenized with sodium phosphate buffer. After added 5 ml of the purified 'cold' TMV (2.5 mg/ml) 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 5 ml of the same buffer and layered on the top of the column. TMV fraction was eluted by the same buffer at room temperature. Each 4 ml of the effluent was assayed for its UV absorption at 260 nm. From each fraction, 0.5 ml was put into a counting vial with 10 ml of Bray's scintillator1). The radioactivity was measured by a liquid scintillation spectrometer.

Chemicals Citrinin was purified by finally adding ethanol after the isolation from the culture of Penicillum sp. AK-0197). Ten mg of citrinin was dissolved in 1.0 ml of acetone and diluted to a given concentration with distilled water. 14C-leucine (specific activity 55.2 mCi/mM) and Ba14CO₃ (specific activity 52.0 mCi/mM) were purchased from The Radiochemical Centre, Amersham, England.

Results and discussion

Recovery of 14C-labeled TMV detected by agar-gel filtration Two fresh leaves of N. glutinosa were homogenized by grinding with mortar and pestle in 3 ml of 0.2 M sodium phosphate buffer (pH 7.0) in the cold at 4°C. The homogenate was centrifuged at 12,000 rpm for 30 minutes. The mixture of 3 ml of the supernatant and 3 ml of TMV solution (OD260 : 27), which contained 1 ml of 14C-labeled TMV, was layered on the top of the column. Agar-gel filtration pattern, shown in Fig 2, had two distinct peaks in optical density at 260 nm. Peak I eluted fast corresponded to TMV, according to the identical position of the radioactivity of 14C-labeled TMV added as a marker. In this case, amount of TMV chromatographed was confirmed to be almost completely recovered by
calculating the total optical density values of virus effluent. A large peak II was yellowish UV-absorbing substances of the supernatant from *N. glutinosa* leaves.

![Fig. 2](image1.png)

Fig. 2. Agar-gel filtration pattern of the mixture of 14C-labeled TMV and the supernatant of the homogenate from *N. glutinosa* leaves.

The mixture of 3 ml of the supernatant and 3 ml of TMV solution (OD260: 27), which contained 1 ml of 14C-labeled TMV as a marker, was chromatographed. Filled circles: optical density at 260 nm, open circles: 14C-radioactivity, peak I: TMV, peak II: leaf component.

![Fig. 3](image2.png)

Fig. 3. Separation of 14C-labeled TMV fraction from infected and 14CO2-exposed *N. glutinosa* leaves by agar-gel filtration.

Infected thirty-six leaves were exposed to 14CO2 for 48 hours from 24 hours after inoculation. After TMV was added as a carrier, TMV solution was prepared by the modified Taniguchi's method with 3 cycles of isoelectric precipitation. Symbols: see Fig. 2.

**Separation of radioactive TMV synthesized in 14CO2-exposed *N. glutinosa* leaves by agar-gel filtration**

Thirty-six detached leaves of *N. glutinosa* were exposed to 14CO2 for 48 hours from 24 hours after inoculation with 0.5 mg/ml of TMV at 25 C under 10,000 lux. TMV preparation obtained from the leaves, on which 196 necrotic lesions in average were produced, by the modified Taniguchi's method with three cycles of isoelectric precipitation in order to refine as much as possible, was chromatographed on the same column. The result is given in Fig. 3. Radioactive TMV (peak I) was eluted from the column in fraction numbers 15-20 and could be completely separated from the leaf component (peak II), which was fractionated as a small radioactive peak. This distinct fractionation is consistent with the result in Fig. 2. In this case, the rate of recovery of virus was 167% of 'cold' TMV added as a carrier, thus showing that 14C-labeled TMV corresponded to at least 67% of the amount of 'cold' TMV, was newly synthesized during 14CO2-exposure. This fact suggests that 14CO2 fixed through photosynthesis abundantly incorporates into TMV synthesized in *N. glutinosa*, similarly as in the tobacco leaves.

As have been reported, 14C-labeled TMV was fractionated by agar-gel filtration when 14C-glycine was infiltrated through the petiole of infected *N. glutinosa* leaves. The 14CO2 fixed through photosynthesis in leaves incorporates into the pool of free amino acids from which TMV is synthesized.
The \(^{14}\text{C}\)-labeled TMV yield would be increased in proportion to the increase in the pool \(^{14}\text{C}\) level. In conclusion, the present method can be utilized successfully in the study of TMV multiplication in \(N.\text{ glutinosa}\) leaves.

**Effect of citrinin on local lesion formation and TMV multiplication in \(N.\text{ glutinosa}\) leaves**

Ten to twelve detached leaves of \(N.\text{ glutinosa}\) were exposed to \(^{14}\text{CO}_2\) for 48 hours immediately after inoculation with 0.1 mg/ml of TMV, infiltrating 25 ppm citrinin solution through the petiole into leaves. Relative number of local lesion formed on citrinin-treated leaves was 1.4% of that of untreated ones. As shown in Fig. 4, the radioactivity of \(^{14}\text{C}\)-labeled TMV from the same treated leaves decreased by about 20% of the untreated. In this case, the amount of citrinin infiltrated in \(N.\text{ glutinosa}\) leaves for 48 hours through the petiole during \(^{14}\text{CO}_2\)-exposure, was estimated to be 3-4 times of that in the leaves treated under the atmospheric pressure\(^6,7\), because an air-pressure in the chamber was reduced owing to the adding of \(^{14}\text{CO}_2\), thus strongly inhibiting both local lesion formation and virus multiplication even at the low concentration, 25 ppm. The inhibitory effect of citrinin on local lesion formation far exceeded that on virus synthesis in all experiments.

It is unknown whether or not citrinin acts on photosynthetic \(\text{CO}_2\) fixation.

In the case of 24 hours' \(^{14}\text{CO}_2\)-exposure to the leaves treated with 10 ppm citrinin immediately or 24 hours after inoculation, effects of citrinin were also recognized, as given in Fig. 5. Even at 24-hour infection stage when local lesions...
did not appear, TMV has already been synthesized in *N. glutinosa* leaves in some degree (Fig. 5a). Radioactive virus was also detected in the leaves treated with citrinin during 0–24 hours after inoculation, but the virus content decreased by 20% of the untreated (Fig. 5b). On the other hand, the amount of TMV synthesized in the leaves developing local lesions at 48-hour increased twenty times as much as that at 24-hour infection stage, when leaves were exposed to $^{14}$CO$_2$ at 24 hours after inoculation (Fig. 5c). It seems that the virus rapidly multiplied concomitantly with the lesion production during 24–48 hours after inoculation. In the case of citrinin-treatment, $^{14}$C-labeled TMV content and the number of local lesions were 40 and 1% of the untreated, respectively (Fig. 5d). These data show that even when treated 24 hours after inoculation, citrinin strongly inhibited local lesion formation, whereas the inhibitory effect on virus multiplication decreased in comparison with the treatment at the time immediately after inoculation. Citrinin may act considerably on virus synthesis when treated during the early infection stages, by the different way from the inhibitory mechanism against local lesion formation on *N. glutinosa* leaves.

**Literature cited**


**和文 摘要**

シトリニンで処理した感染グルチノーザ葉内で増殖した

TMV の寒天グルテーザンによる分離

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$^{14}$CO$_2$ で処理したタバコモサイクウイルス (TMV) 感染グルチノーザ葉から，$^{14}$C-標識 TMV を寒天グルテーザン過法で分離した。光合成によって同化された$^{14}$CO$_2$ は有効に同葉内の TMV にとりこまれた。この方法を用いて，シトリニンのグルチノーザ葉内で TMV 増殖に対する作用を検討した。接種直後から48時間，25 ppm のシトリニンを処理したグルチノーザ葉上に形成された局部病変数は無処理の14%で，同葉内で増殖した$^{14}$C-標識 TMV の放射能は無処理の20%に減少した。シトリニンは接種後24時間してから処理しても局部病変形成を阻止したが，ウイルスの増殖に対しては主として感染初期に作用することが暗示された。