日植病報 42：450-455 (1976)
Ann. Phytopath. Soc. Japan 42：450-455 (1976)

Isolation and Identification of a Substance Interfering with Local Lesion Formation Produced in Cowpea Leaves Locally Infected with Cucumber Mosaic Virus

Sakari KATO* and Tadao MISAWA*

加藤　盛*・三沢正生*：キュウリ・モザイク・ウイルス感染ササゲ葉で生産される局部病斑形成阻害物質の分離と同定

Abstract

A substance, undetectable in uninfected leaves, was isolated from cowpea leaves locally infected with cucumber mosaic virus (CMV). This substance inhibited the formation of local lesions caused by CMV on cowpea leaves and it was identified as traumatic acid, which has been specified as a wound hormone of plants, on the basis of both chemical properties as well as physiological action.

(Received January 17, 1976)

Introduction

Gilpatrick and Weintraub[3] first observed that infection in one part of plants induced resistance in other noninvaded tissues. They did not find the presence of the virus in the upper uninfected leaves of a clone of *Dianthus barbatus*, of which lower leaves had been inoculated with carnation mosaic virus[15]. Yarwood[16, 17] and Ross[11, 12] reported that leaf tissues near the local lesions induced by tobacco mosaic virus (TMV), were generally resistant to reinoculation. These phenomena were called as "localized acquired resistance". A similar phenomenon was found in CMV-infected cowpea[10]. These facts suggest the presence of some antiviral substances which act in advance of the occurrence of cell necrosis. Loebenstein *et al.*[7, 8, 9], working with TMV and *Datura stramonium*, postulated that the virus-interfering agent was a protein-like substance of low molecular weight. Sela *et al.*[13, 14], using TMV and *Nicotiana glutinosa*, reported that the antiviral factor is associated with RNA rather than protein, and it differs from interferon because of lack of host specificity. Kimmins[6] found a low molecular weight RNA as an active principle in the resistant tissues of *D. stramonium* and in other plants, which could be detected by the inoculation of other parts with TMV or tobacco necrosis virus (TNV). We[4] previously demonstrated the presence of antiviral or interfering agents with a low molecular weight in cowpea leaves infected with CMV.

This paper describes the procedure of isolation and identification of a crystalline active substance which was produced in cowpea leaves locally infected with CMV.

* Faculty of Agriculture, Tohoku University, Sendai, Japan 東北大学農学部

Materials and Methods

Test plants. Cowpea plants (*Vigna sinensis* Endl. *var. sesquipedalis*, cultivar Kurodane-sanjaku) were grown in a green house controlled at 25 C. The upper surface of a primary leaf, at the primary leaf stage, was rubbed with a yellow strain of CMV. The opposite leaf was rubbed with 0.1 M phosphate buffer, pH 7.0, to serve as a control. Leaves were harvested 24 hr after inoculation for the isolation of a crystalline active substance. Lesion numbers produced per inoculated leaf were 600 or more.

Isolation procedure of crystalline active compound. Infected and uninfected leaves were ground separately, and were immediately extracted with water at 50-60 C. The extract was concentrated under reduced pressure to a syrup. The syrup was acidified with HCl to pH 2 and then extracted repeatedly with acetone. The acetone extract was evaporated to dryness at 60 C and the residue was dissolved in water. Then the solution was exhaustively extracted with ethyl acetate after adjusting pH at 2.0. The extract was subjected to evaporation as before, and the resulting oily residue was dissolved in a small amount of dilute NaOH. Further, it was extracted with chloroform and the water layer was acidified to pH 2.0, and again extracted with chloroform. After evaporating chloroform, the residue was dissolved in a small amount of 0.1 M NaOH and the solution was added with 20 % solution of $BaCl_2$ until a precipitate was produced. After centrifugation, the supernatant, a soluble barium salt fraction, was acidified with dilute HCl and extracted with ethyl acetate. Ethyl acetate was evaporated and then was obtained oily substance which partially crystallized on standing. The crystalline substance was separated by the addition of isopropyl ether and recrystallized from absolute alcohol.

Infrared, Nuclear magnetic resonance and Mass spectra analysis of isolated compound. Infrared (IR) spectra were measured in KBr pellet with Infrared spectrophotometer (Hitachi model 295). Nuclear magnetic resonance (NMR) spectra were measured in d-6 dimethyl sulfoxide solution with magnetic resonance apparatus (Hitachi R-24A, 60 MHz). Mass spectra were obtained with Mass spectrometer, Hitachi type M-52.

Results and Discussion

Biological activity of isolated compound.

A crystalline substance was obtainable from infected cowpea leaves, but not from uninfected leaves. Yield was about 0.1 mg per Kg of fresh leaves. This substance had no significant effect on the virus infectivity when it was mixed with CMV prior to inoculation of cowpea leaves (Table 1). Therefore, the biological activity of the substance was assayed by floating method as follows: cowpea or tobacco leaves (*Nicotiana tabacum* L. cultivar Ky 57) inoculated with CMV were floated on solutions containing 1, 10, 50 or 100 ppm of the crystalline substance. Distilled water and oily material fraction obtained from uninfected leaves were used as a control, because the crystalline substance could not be separated from the oily material fraction of uninfected leaves. As shown in Table 1, the number of local lesions on cowpea leaves was markedly reduced at 50 ppm of the crystalline substance.

A conspicuous difference was also noticed in the size of local lesions. The cowpea leaves floated on the crystalline substance of 50 and 100 ppm, produced lesions ranging from 0.2-0.6 mm in diameter, with the mode of 0.2-0.3 mm (out of sample of 100 lesions), whereas the control leaves, floated on water or oily material

Table 1. Effect of the crystalline substance isolated from CMV-infected cowpea leaves on lesion formation on cowpea leaves when either mixed with CMV or floated on solution immediately after CMV inoculation

Concentration of crystalline substance (ppm)	Average number of local lesions per leaf[a]	
	mixed with CMV [b]	floating[c]
0	209	202
1	212	191
10	201	155
50	216	103
100	197	23
Oily material suspension from uninfected leaves [d]	204	199

a) Average of 10 leaves in 3 separate experiments.
b) Crystalline substance mixed with CMV was inoculated on cowpea leaves. They were floated on distilled water for 3 days.
c) Inoculated leaf disks were floated on water solution of the crystalline substance at different concentrations.
d) The cencentration was 10 mg/ml.

Table 2. Effect of isolated crystalline substance on CMV multiplication in tobacco leaf disks

Concentration of crystalline substance (ppm)	Average number of local lesions per leaf[a]	
	mixed with CMV[b]	floating [c]
0	153	189
1	164	183
10	143	193
50	158	197
100	144	180
Oily material suspension from uninfected leaves[d]	144	185

a) Average of 10 leaves in 3 separate experiments. Lesions were counted 24 hr after inoculation.
b) Tobacco leaf disks (12 mm in diameter) inoculated with CMV mixed with crystalline substance were floated on distilled water.
c) Tobacco leaf disks inoculated with CMV alone were floated on aqueous solutions containing various concentrations of crystalline substance.
d) The concentration was 10 mg/ml.

fraction obtained from uninfected leaves, developed lesions ranging from 0.4-0.8 mm in diameter, with a mean of 0.6 mm. However, this crystalline substance had no effect on CMV multiplication in tobacco leaves (Table 2). Since this substance is undetectable in uninfected leaves, it seems to be synthesized *de novo* in the infected cowpea leaves. The crystalline substance therefore may not directly inhibit virus multiplication, but exert its inhibitory effect through a metabolic change of cowpea leaves.

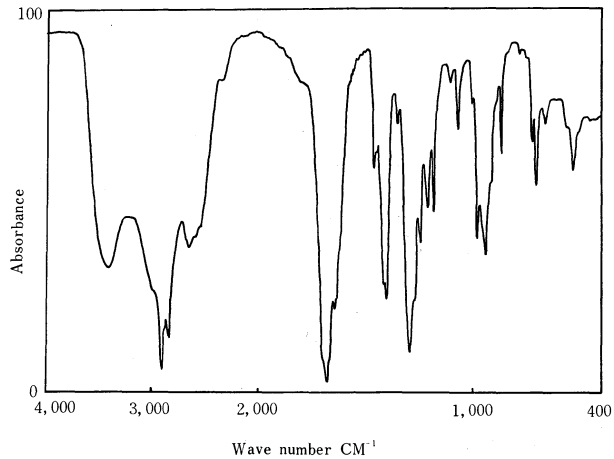

Fig. 1. Infrared spectrum of the crystalline substance isolated from CMV-infected cowpea leaves in KBr pellet.

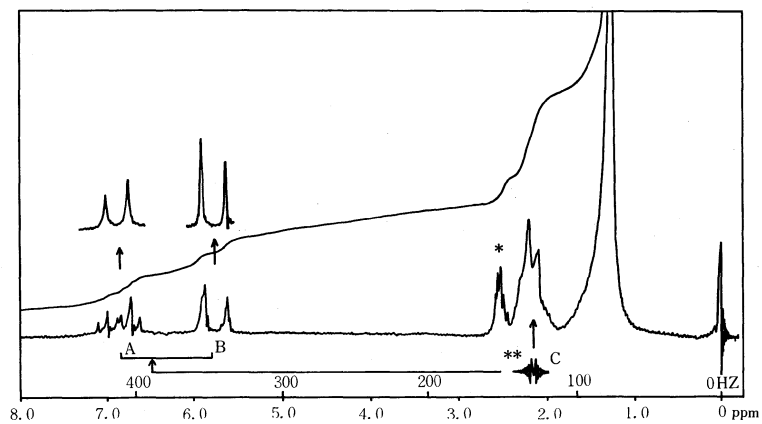

Fig. 2. Nuclear magnetic resonance spectrum of the crystalline substance isolated from CMV-infected cowpea leaves. The analysis was carried out in d-6-dimethyl sulfoxide solution and tetramethylsilane was used a reference.

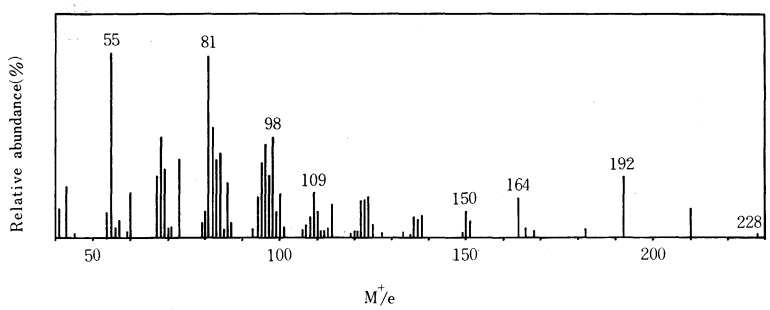

Fig. 3. Mass spectrum of the crystalline substance isolated from CMV-infected cowpea leaves. Mass spectrum was obtained with mass spectrometer operating at an ionizing potential of 20 eV; ion source temperature, 200 C; direct inlet.

Identification of isolated compound

For identification of this substance, IR, NMR and Mass spectra analysis were carried out. The results of analysis are depicted in Fig. 1, 2 and 3. The IR spectrum of the isolated substance indicated the presence of a trans conjugated double bond (940 and 980 cm^{-1}). The peaks at 1680 cm^{-1} and 2500-3700 cm^{-1} indicated a carboxyl group. The NMR spectrum of this substance was made up as follows: δ 1.28 [12H, singlet, $-(CH_2)_6-$], δ 2.0-2.4 (4H, broad doublet, $-CH_2-$), δ 5.73 (1H, doublet, J=15.6 Hz, $-\overset{|}{C}=\underset{H}{C}-COO$), δ 6.83 (1H, triplet of doublets, J=7.0 and 15.6Hz, $\overset{H}{C}-C=\overset{O}{\overset{\|}{C}}-$). The mass spectrum of this substance showed a small parent peak (M^+/e 228). From these results, the structural formula of this substance is represented by $HOOC-CH_2-(CH_2)_6-CH_2-\overset{H}{C}=\overset{H}{C}-COOH$, trans form. It had a molecular weight of 228, and m. p. 163-164 C. The formula of this crystalline substance coincides with that of traumatic acid, which has been specified as a wound hormone of plants[1]. The isolated compound was, therefore, subjected to standard bean test[1] in order to ascertain its hormonal activity. It exerted hormonal activity when glutamic acid (0.1-0.2%) was added as co-factor in the assay system. On the basis of these similarities, it is concluded that the crystalline substance, extracted from CMV-infected cowpea leaves, is traumatic acid. Consequently, it appears that traumatic acid not only had a wound healing action as reported by English *et al.*,[1] but also interferes with local lesion formation in cowpea leaves.

Traumatic acid has been reported to be derived from peroxidation of lipids by lipoxygenase[2]. In fact, lipoxygenase activity increased in CMV-infected cowpea leaves, and the peroxidation of lipids by this enzyme seemed to play an important role in the formation of hypersensitive lesions as well as in the limitation of CMV spread[5].

These facts suggest that traumatic acid produced after CMV infection is likely involved in the limitation of formation and growing of local lesions in cowpea leaves.

Literature cited

1) English, J. Jr., Bonner, J. and Haagen-Smit, A. J.(1939). Proc. Natl. Acad. Sci. U.S.A. 25: 323-329.
2) Fujino, Y. (1972). *In* Course of Plant Physiology 2 (Furuya, M., Miyaji, S. and Kumura, A. *eds.*). Asakura, Tokyo. pp 231-232.
3) Gilpatrick, J. D. and Weintraub, M. (1952). Science 115: 701-702.
4) Kato, S. and Misawa, T. (1974). Ann. Phytopath. Soc. Japan 40: 205-206.
5) Kato, S. and Misawa, T. (1975). *Ibid.* 41: 295.
6) Kimmins, W. C. (1969). Can. J. Bot. 47: 1879-1886.
7) Loebenstein, G. and Ross, A. F. (1963). Virology 20: 507-517.
8) Loebenstein, G. and Tirzah van Praagh (1964). *In* Host-parasite relations in plant pathology (Király, Z. and Ubrizsy, G. *eds.*) Budapest. pp53-58.
9) Loebenstein, G. (1972). Ann. Rev. Phytopath. 10: 177-206.
10) Okamoto, S., Kato, S. and Misawa, T. (1968). Ann. Phytopath. Soc. Japan 34: 361.
11) Ross, A. F. (1959). Phytopathology 49: 549.
12) Ross, A. F. (1961). Virology 14: 329-339.
13) Sela, I., Harpaz, I. and Birk, Y. (1964). *Ibid.* 22: 446-451.
14) Sela, I., Harpaz, I. and Birk, Y. (1966). *Ibid.* 28: 71-78.
15) Weintraub, M. and Kemp, W. G. (1960). *Ibid.* 13: 256.
16) Yarwood, C. E. (1953). Phytopathology 43: 490.
17) Yarwood, C. E. (1960). *Ibid.* 50: 741-744.

和文摘要

キュウリ・モザイク・ウイルス感染ササゲ葉で生産される局部病斑形成阻害物質の分離と同定

加藤　盛・三沢正生

キュウリ・モザイク・ウイルス（CMV）に感染，局部病斑を形成しているササゲ初生葉から一種の結晶性物質を分離した。本物質は健全葉では見出し得ないことから，感染によって新たに生産されるものと思われる。この物質を外部から与えた場合，ササゲでのCMVによる局部病斑の形成は抑制される。化学的および生理学的諸性質から，本物質は植物の癒傷ホルモンと呼ばれるトラウマチン酸と同定された。