A Potent Plant Virus Inhibitor Found in *Mirabilis jalapa* L.

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Abstract

An inhibitor of plant virus infection was found in the extracts of *Mirabilis jalapa* L. The inhibitor, a protein designated as MAP (*Mirabilis* antiviral protein), showed highly potent activity against mechanical transmission of viruses, tobacco mosaic (TMV), cucumber green mottle mosaic, potato Y, turnip mosaic and cucumber mosaic. Almost complete inhibition was achieved when MAP at 0.8 µg/ml was applied on the upper surface of leaves of Xanthi nc tobacco 24 hr before TMV inoculation, and 50% inhibition when MAP at 10 µg/ml was applied on the under surface of leaves. MAP induced systemic resistance to TMV infection in tobacco plants when it was applied on their basal leaves 24 hr before TMV inoculation onto the upper ones. MAP showed no inhibition when it was applied 1 hr after TMV inoculation. Antiserum against MAP gave positive reaction in the agar gel with leaf extracts of *M. jalapa* and did not with those of the other Chenopodiales plants such as *Basella rubra*, *Boerhaavia diffusa*, *Bougainvillea* sp., *Chenopodium amaranticolor* and *Phytolacca americana*. Concentration of MAP was successfully quantified using enzyme-linked immunosorbent assay. MAP content in the root tissue was exceedingly higher than in the other parts of the plant and that of an yellow-flower trait of *M. jalapa* was as high as 1.1 mg/g fresh weight. *M. jalapa* seems to be a promising source of antiviral substances for the practical use.

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Key words: *Mirabilis jalapa*, anti-plant viral protein, virus inhibitor, tobacco mosaic virus, tobacco.

INTRODUCTION

It is well recognized that a number of higher plants contain inhibitory substances against virus infection, and a few of potent inhibitors in the plants have been isolated and characterized^{2,5,13,14,20,24}). Although studies on the distribution of inhibitors in the plant kingdom and on the mode of action have been done^{3,7,12,15-17,25}), the inhibitors in plants have also drawn attention as a source of antiviral substances for the practical use¹¹). During the course of screening works on the antiviral substances, we found that the plant *Mirabilis jalapa* L. (four-o'clock flower) contains a potent inhibitor of plant virus infection. The inhibitor was proved to be a

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basic protein and referred to as MAP (*Mirabilis* antiviral protein). This paper describes the inhibitory behaviors of MAP on plants, MAP content in *Mirabilis* plants and serological relationship of MAP to the other antiviral components having found in the related plant species. A purification procedure and chemical characterization of MAP will be described in the next paper¹⁸⁾.

A part of this study has been previously presented^{9,10)}.

MATERIALS AND METHODS

Viruses. An ordinary strain of tobacco mosaic virus (TMV)⁴⁾, cucumber strain of cucumber green mottle mosaic virus (CGMMV)⁸⁾, a necrotic strain of cucumber mosaic virus (CMV-R1, unpublished), a necrotic strain of potato virus Y (PVY)²¹⁾ and turnip mosaic virus (TuMV)¹⁹⁾ were used to test the antiviral activity of the inhibitor. Purified preparations of TMV, CMV and PVY and leaf extracts of CGMMV-infected cucumber and TuMV-infected turnip plants were used as inocula.

Plants. At the early stage of the present work, *M. jalapa* plants were obtained at gardens in Kawasaki and Yokohama. Later, seed lots collected from various places in Japan and outside were grown in the greenhouse and/or fields. Tobacco (*Nicotiana tabacum* L.) cvs. Bright Yellow, Burley 21, Xanthi nc, *Datura stramonium* L., tomato (*Lycopersicon esculentum* Mill.) cv. Ponterosa, pepper (*Capsicum frutescens* L.) cv. Takanotsume, cucumber (*Cucumis sativus* L.) cv. Sagamihanjirō were used as the assay hosts.

Inhibitor source. Fresh tissues of leaves, stems and roots of *Mirabilis* plants were ground in a mortar and pestle with 10 mM Na-phosphate buffer (PB), pH 7.2, containing 0.1 % mercaptoethanol (PBM). The homogenates were strained through a layer of Miracloth (CAL-BIOCHEM). Acetone and ammonium sulfate precipitates of the root extracts, and purified MAP were also used as the inhibitor sources. Lyophilized root tissues were ground in a Waring blender and then homogenized with 20 volumes (v/w) of 10 mM PBM for 5 min. The homogenate was centrifuged at $5,000 \times g$ for 15 min and the supernatant was collected. The precipitate was resuspended with 10 volumes of PBM and centrifuged again and the resulting supernatant was combined with that of the previous centrifugation. To the supernatant acetone was added to 40% and stirred for 30 min. The suspension was centrifuged at $5,000 \times g$ for 15 min and the resulting precipitate was lyophilized and stored in a desiccator until use. The ammonium precipitate and purified MAP were obtained according to the method as described¹⁸⁾.

Bioassay of the inhibitory activity. The antiviral effect of the inhibitor was assayed by the half leaf method using local lesion hosts of the viruses. The virus-host combinations were TMV-Xanthi nc tobacco, CGMMV-*D. stramonium*, CMV-Bright Yellow tobacco and TuMV-Bright Yellow tobacco. The inhibitor was applied with a paintbrush to half leaves of the under or upper surface of expanded ones while water was applied onto the opposite half leaves as the control. Unless otherwise stated, the viruses were inoculated onto the upper surface of the test plants 24 hr after the inhibitor treatment. The number of local lesions appeared were counted 3 to 7 days after inoculation and percent inhibition was calculated according to the following formula: $(1 - T/C) \times 100$, where C is the number of local lesions on the control half leaves and T is the number of local lesions on the treated halves.

In another experiment, the inhibitor was sprayed with a manual atomizer on to the whole surface of 5 basal leaves of Xanthi nc tobacco and TMV was inoculated onto 3 upper-untreated leaves 1 or 3 days after the treatment to know the systemic effects of the inhibitor.

Serology. An antiserum against MAP was obtained by injecting a rabbit with the purified MAP (0.78 mg/0.5 ml) emulsified with an equal volume of Freund's incomplete adjuvant. The initial injection was made to the foot pads followed by the same amount of MAP to the back 2 weeks later. The antiserum obtained 3 weeks after the final injection had a titer of 1:512 in agar gel diffusion tests. Tests for serological relationships between MAP and components

from various plant species were done by agar gel diffusion tests using 0.8% agar (Difco) in 10 mM PB containing ethylenediaminetetraacetic acid (PBE), pH 7.2. Extracts of leaves were prepared by grinding in a mortar 50 mg of lyophilized laminae with 2.5 ml of PBE and by squeezing through a layer of Miracloth.

Enzyme-linked immunosorbent assay (ELISA). The procedures used in the test were essentially as described by Clark and Adams¹⁾ except for the following modifications. The microtiter plates (Nunc-Immuno Plate I, Nunc, Denmark) were coated with γ -globulin at 5 μ g/ml by incubating for 6 hr at 25 C or overnight at 4 C. Tissues of *M. jalapa* were triturated using a mortar and pestle in 20 mM PB (10 ml/g tissue), pH 7.4, containing 0.15 M NaCl, 0.05% Tween 20, and 0.1% 2-mercaptoethanol (PBSTM), and then centrifuged at 3,000 rpm for 10 min. The tissue extracts were serially diluted with PBSTM, added to the wells, and incubated overnight at 4 C. Purified MAP was also added at 111.1 to 1.37 ng/ml to each microtiter plate to serve the standard dilution curve.

RESULTS

Inhibitory activity of extracts from M. jalapa

Effect of crude extracts from *M. jalapa* plants on virus infection was tested in Xanthi nc tobacco. The results showed that the crude extracts at a 1/50 dilution were strongly inhibitory to TMV infection, almost 100% inhibition at the upper surface treatment and 50% or more even at the under surface (Table 1).

Inhibitory activity of the root extract and its ammonium sulfate precipitate was also observed in several virus-host combinations (Table 2). In addition to the results obtained in the

Table 1. Effect of crude extracts from *Mirabilis jalapa* on the infectivity of tobacco mosaic virus ^{a)}

Source of extract		Leaf surface treated	No. of local lesion/half leaf ^{b)}		Inhibition (%)
			Control	Treated	
Root	1/50	Under surface	120.5	50.8	58
	1/5	"	147.2	21.7	85
Leaf	1/50	"	194.2	96.0	51
	1/5	"	161.0	43.3	73
Root	1/50	Upper surface	130.8	0.3	99.8

a) TMV (0.05 μ g/ml) was inoculated onto upper surface of leaves 24 hr after treatment.

b) Mean no. of local lesions on 6 half leaves of Xanthi nc tobacco.

Table 2. Inhibitory activity of *Mirabilis* extracts in several virus-host combinations

Virus ^{a)}	Host plant ^{b)}	Preparation treated	Concentration (mg/ml)	Leaf surface treated	Inhibition (%)
TMV	Tobacco (Xanthi nc)	Acetone precipitate	1.0	Under surface	72
CMV	Tobacco (Bright Yellow)	"	1.0	"	95
"	"	"	0.1	Upper surface	100
TuMV	"	"	1.0	Under surface	75
"	"	"	0.1	Upper surface	97
CGMMV	<i>D. stramonium</i>	Root extracts ^{c)}	1/25	Under surface	54
"	"	"	1/500	Upper surface	98

a) Viruses were inoculated 24 hr after treatment. The concentrations of the inocula of TMV, CMV and PVY were 0.05 μ g/ml, 5 μ g/ml and 5 μ g/ml, respectively, while 1/5,000 and 1/100 dilute solutions of extracts from infected leaves were employed for CGMMV and TuMV, respectively.

b) Six leaves were used for each test.

c) Extract of fresh roots was diluted to 1/25 to 1/500.

local lesion hosts, the inhibitory activity was also proved in systemic hosts. Preinoculation spray of a solution of the acetone precipitate at 1 mg/ml completely inhibited PVY infection in Burley tobacco, although all of the untreated plants were infected by the mechanical inoculation with the same virus inoculum. Similar results were obtained in TMV-tomato and pepper, and CGMMV-cucumber plants (data not shown).

Systemic effect of the inhibitor

Since the extract of *M. jalapa* and its precipitate applied to the under surface of leaves showed inhibitory activity to virus infection, an experiment was carried out to examine whether the inhibitory activity was exhibited systemically or not. As shown in Table 3, the number of local lesions appeared on the treated plants decreased to about a half of those on the water-sprayed control.

Dose-response curve of MAP

When MAP was applied onto the under surface of tobacco leaves, the inhibitory activity was observed at concentrations higher than 4 μ g/ml, and 50% inhibition dose (ID_{50}) was estimated to be around 10 μ g/ml (Fig. 1). The activity of MAP was exhibited much markedly when it was applied onto the upper surface of leaves, the same surface of leaves where TMV was inoculated. Almost 100% inhibition was achieved at the concentration of MAP as low as 0.8 μ g/ml.

Effect of the time of treatment

To see the mode of action of MAP, treatment with MAP was made before or after TMV

Table 3. Systemic inhibitory effect of *Mirabilis* extracts on the infection of tobacco mosaic virus^{a)}

Leaf position	No. of lesions/leaf		
	Untreated	Treated plant	
		1 day after treatment	3 days after treatment
14	596.3 (100) ^{b)}	274.8 (46)	290.8 (49)
13	447.3 (100)	212.3 (48)	236.5 (53)
12	521.0 (100)	285.3 (55)	301.8 (58)
11	516.3 (100)	0.3 (0.1)	0 (0)

- a) An acetone precipitate of root extracts of *M. jalapa* (2 mg/ml) was sprayed onto whole surface of 5 basal leaves (7th to 11th above soil level) of Xanthi nc tobacco and then TMV was inoculated on 3 upper-untreated leaves (12th to 14th) and on a treated leaf (11th) 1 or 3 days after treatment. Untreated (control) plants were sprayed with water. Two plants were used for each test.
- b) Figures in parentheses represent relative no. of local lesions as the no. of lesions on the respective control leaf being 100.

Fig. 1. Effect of various concentrations of purified MAP on tobacco mosaic virus infection of Xanthi nc tobacco. MAP was applied onto the upper surface (●) or under surface (○) of tobacco leaves 24 hr after inoculation.

inoculation. MAP was applied onto the under surface of leaves of Xanthi nc tobacco at a concentration of 100 $\mu\text{g/ml}$. As shown in Table 4, there was no appreciable inhibition even when it was applied one hr after inoculation, although there observed the marked inhibition at every treatment before inoculation.

Serological studies

An antiserum against MAP formed a single precipitin line with a purified preparation of MAP in agar gel diffusion tests, and the detection end point of MAP was 0.5 $\mu\text{g/ml}$. Using the anti-MAP serum, serological relationships between leaf extracts of *M. jalapa* and those of several plants known to contain antiviral substances were examined in the agar gel. The antiserum gave the positive reaction only with the leaf extract of *M. jalapa* and did not with those of other plants as shown in Fig. 2.

MAP content in *M. jalapa*

The concentration of MAP was successfully assayed by the ELISA technique and was able to be quantified in a range between 4 and 111 ng/ml using absorbance values at 405 nm, though detectable concentration of MAP was less than 4 ng/ml.

An yellow-flower trait of *M. jalapa* collected at Hatano, Kanagawa Prefecture was grown in the greenhouse for 3 months after sowing and MAP content in various parts of the plant was assayed by ELISA. MAP content in flower petals, leaves, stalks and roots was 0.7, 2.1–3.0, 7.4–51.8 and 492.8 $\mu\text{g/g}$ fresh weight, respectively.

Table 4. Effect of MAP treatment to the under surface of Xanthi nc tobacco leaves before or after inoculation with tobacco mosaic virus ^{a)}

Time of treatment (hr)	No. of local lesions/half leaf ^{b)}		Inhibition (%)
	Untreated	Treated	
–24	134.0	59.0	56
–6	290.7	58.2	80
–3	255.8	20.8	92
–1	336.0	40.8	88
+1	207.0	180.7	13
+6	132.3	162.7	–23
+24	115.7	126.3	–9

a) Purified MAP at 100 $\mu\text{g/ml}$ was applied at the indicated time (hr) before (–) or after (+) virus inoculation.

b) Mean no. of local lesions on 6 half leaves of Xanthi nc tobacco.

Fig. 2. Agar gel diffusion tests with the antiserum to MAP and leaf extracts from 6 Chenopodiales plants. Inner wells contained the antiserum diluted to 1:128. Peripheral wells contained purified MAP at 5 $\mu\text{g/ml}$ (1 and 5) and leaf extracts from *Mirabilis jalapa* (2), *Bougainvillea* sp. (3), *Boerhaavia diffusa* (4), *Phytolacca americana* (6), *Basella rubra* (7) and *Chenopodium amaranticolor* (8).

DISCUSSION

The present study definitely shows that the plant *M. jalapa* contains a potent inhibitor (MAP) of virus infection. *M. jalapa* belongs to the family Nyctaginaceae, which includes the inhibitor-containing plants, *Boerhaavia diffusa*^{22,23)} and *Bougainvillea spectabilis*¹¹⁾. It has been pointed out that plant species belonging to the order Chenopodiales and to the subclass Centrospermae widely contain virus inhibitors^{3,16)}. Indeed, *M. jalapa* belongs to the Chenopodiales, and MAP of *M. jalapa* is a basic protein¹⁸⁾, sharing several properties with that of *P. americana*. From the serological study, however, MAP seems to be a protein specific to *M. jalapa*. Since almost 100% inhibition was achieved by the preinoculation treatment to the upper leaf surface at 0.8 µg/ml (Fig. 1), the specific inhibitory activity of MAP is comparable to or much higher than that of the protein from *P. americana*^{6,24)}. It is considered that MAP is not the inhibitor of virus synthesis but that of virus infection because no appreciable inhibition was observed when MAP was applied after virus inoculation (Table 4).

A noteworthy feature of MAP is that its inhibitory activity is exhibited systemically. However, it seems unlikely that the proteinaceous inhibitor is capable to penetrate into leaf cells and translocate to long distance in sufficient amount to block virus infection directly. The systemic effect of MAP might be a host-mediated phenomenon.

M. jalapa is an ornamental plant with tuberous roots and there are many traits varying in their flower colors. It was proved by ELISA that MAP is the most concentrated in the roots and that the content in roots is variable from trait to trait. Among several traits or seed lots tested, an yellow-flower trait collected at Hatano contained the highest amount of MAP, up to 1.1 mg/g fresh root (data not shown). Content of antiviral substance in *M. jalapa* is estimated to be much higher than that in carnation¹³⁾ or *P. americana*²⁴⁾. *M. jalapa* seems to be a promising source of antiviral substances for the practical use.

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Literature cited

1. Clark, M.F. and Adams, A.N. (1977). J. gen. Virol. 34: 475-483.
2. Fukaya, N. and Taniguchi, T. (1979). Phytopath. Z. 94: 132-138.
3. Grasso, S. and Shepherd, R.J. (1978). Phytopathology 68: 199-205.
4. Hidaka, Z. and Hiruki, C. (1965). Bull. Hatano Tob. Exp. Sta. 56: 43-57.
5. Irvin, J.D. (1975). Arch. Biochem. Biophys. 169: 522-528.
6. Irvin, J.D., Kelly, T. and Robertus, J.D. (1980). Ibid. 200: 418-425.
7. van Kammen, A., Noordam, D. and Thung, T.H. (1961). Virology 14: 100-108.
8. Komuro, S., Tochihara, H., Fukatsu, R., Nagai, Y. and Yoneyama, S. (1971). Ann. Phytopath. Soc. Japan 37: 34-42.
9. Kubo, S., Takanami, Y., Ikeda, T. and Mikami, Y. (1985). Ibid. 51: 57 (Abstr.).
10. Kubo, S. and Takanami, Y. (1988). Fifth International Congress of Plant Pathol. St. I-3-4 (Abstr.).
11. Mutphy, N.S., Nagarajan, K. and Sastri, A.B. (1981). Indian J. Agric. Sci. 51: 792-795.
12. Owens, R.A., Bruening, G. and Shepherd, R.J. (1973). Virology 56: 390-393.
13. Ragetli, H.W.J. and Weintraub, M. (1962). Ibid. 18: 232-240.
14. Ragetli, H.W.J. and Weintraub, M. (1962). Ibid. 18: 241-248.
15. Simons, J.N., Swidler, R. and Moss, L.M. (1963). Phytopathology 53: 677-683.
16. Smookler, M.M. (1971). Ann. appl. Biol. 69: 157-168.
17. Stirpe, F., Williams, D.G., Onyon, L.J., Legg, R.F. and Stevens, W.A. (1981). Biochem. J. 195: 399-405.
18. Takanami, Y., Kuwata, S., Ikeda, T. and Kubo, S. (1990). Ann. Phytopath. Soc. Japan 56: 488-494.

19. Tochihara, H. (1965). Bull. National Inst. Agric. Sci. C 18: 1-57.
20. Tomaru, K. and Ohkawa, Y. (1975). Ann. Phytopath. Soc. Japan 41: 155-161.
21. Tomaru, K. (1983). Bull. Morioka Tob. Exp. Sta. 17: 87-96.
22. Verma, H.N. and Awasthi, L.P. (1979). Can. J. Bot. 57: 926-932.
23. Verma, H.N., Awasthi, L.P. and Saxena, K.C. (1979). Ibid. 57: 1214-1217.
24. Wyatt, S.D. and Shepherd, R.J. (1969). Phytopathology 59: 1787-1794.
25. Yoshii, H. and Sako, N. (1967). Ann. Phytopath. Soc. Japan 33: 244-252.

和 文 摘 要

久保 進・池田 勉・今泉誠子・高浪洋一・三上洋一：オシロイバナに含まれる強力な抗植物ウイルス物質

オシロイバナ (*Mirabilis jalapa* L.) に強力な抗植物ウイルス蛋白質 (MAP) が含まれていることを見いだした。MAP はタバコモザイクウイルス (TMV)、キュウリ緑斑モザイクウイルス、キュウリモザイクウイルス、ジャガイモ Y ウイルス、カブモザイクウイルスの汁液接種による感染を高率に阻害した。タバコ (*Xanthi nc*) と TMV の系において、接種 1 日前の葉表塗布処理では、MAP 濃度 0.8 $\mu\text{g/ml}$ でほぼ完全な感染阻害を示した。葉裏処理-葉表接種および下位葉処理-上位葉接種でも高率な感染阻害が認められ、MAP がシステミックな抗ウイルス効果を示すことが知られた。MAP はウイルス接種後の処理では無効であった。MAP の抗血清は、抗ウイルス物質を含むことが報告されているアカザ目植物のナハカノコソウ、ブーゲンビリア、ツルムラサキ、*Chenopodium amaranticolor*、ヨウシュヤマゴボウの葉汁液とは反応せず、MAP がオシロイバナ独特の蛋白質であることを示唆していた。MAP の抗血清を用いて ELISA を確立し、オシロイバナに含まれる MAP を定量した。MAP は花、葉、茎、根の各部に含まれるが、根部の含量が最も高く、その量は秦野市で採集した黄花系統の生根で 1.1 mg/g であった。オシロイバナが抗植物ウイルス剤の原料として有望であることを述べた。