Interactions Between Tobacco Mosaic Virus, Pokeweed Antiviral Proteins, and Tobacco Cell Wall

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ABSTRACT

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Precipitation of tobacco mosaic virus (TMV) with antiviral proteins of *Phytolacca americana* (PAP and PAPII) and adsorption of the proteins to the cell wall of *Nicotiana tabacum* 'Xanthi' were studied using fluorescein isothiocyanate-labeled PAP and PAPII. The antiviral proteins conjugated with TMV in 0–60 mM KCl solution. The maximum ratio for PAP/TMV was 700:1 at pH 5 and for PAPII/TMV was 440:1 at pH 6, and these ratios were below 100:1 at pH 2 and pH 9 to 12, respectively. The antiviral proteins adsorbed to the cell wall in 0–200 mM KCl solution, but did not adsorb in 300 mM KCl. However, since the formation of TMV local lesions on *Nicotiana glutinosa* was inhibited by the antiviral proteins in 300 mM KCl (where the conjugation between TMV and the antiviral proteins and the adsorption of the antiviral proteins on the cell are completely dissociated), the interactions are not the necessary condition for TMV inactivation. In low ionic condition, less than 60 mM KCl, the infectivity of TMV may be partially inhibited by precipitation with the antiviral proteins.

Pokeweed antiviral proteins (PAP) have been studied by many workers. Duggar and Armstrong (5) found that the juice of *Phytolacca decandra* L. inactivates TMV. Kassanis and Kleczkowski (11) found that a glycoprotein obtained from the sap of *Phytolacca esculenta* Van Houtte inhibits tobacco mosaic virus (TMV) infectivity. Wyatt and Shepherd (24) isolated a protein of 13,000 mol wt from *Phytolacca americana* L. that was a virus inhibitor. Irvin (9) purified an antiviral protein of 27,000 mol wt from young plants of *P. americana* growing in early spring, and Irvin et al (10) found a second antiviral protein of 30,000 mol wt from the same plants in the summer season. Since then, an antiviral protein (PAP-S) has been found in the seed of *P. americana* (1) and a ribosome-inhibiting protein in *Phytolacca dodecandra* L'Hér (17). These pokeweed antiviral proteins are inhibitors of in vitro polypeptide synthesis employing rabbit reticulocytes and plant ribosomes (1,2,4,8–10,14–17). Ussery et al (23) indicate that the transport of an antiviral protein from *P. americana* into a HeLa cell depends on the presence of poliovirus, and the invading protein could inhibit protein synthesis in the cell.

When a mixture of TMV and pokeweed antiviral proteins is rubbed on the leaf surface of *Nicotiana glutinosa* L. or *Phaseolus vulgaris* L., very few local lesions appear compared with an inoculum of untreated TMV (7,10,11). A direct action of pokeweed antiviral proteins on viruses in the mixture (the conjugation between virus and antiviral protein) has been reported by several workers. Kassanis and Kleczkowski (11) previously found that a virus-inhibiting glycoprotein from *P. esculenta* can conjugate with TMV, and they indicated that the conjugation is not enough to explain the TMV inactivation. Tomlinson et al (22) showed that cucumber mosaic virus and influenza virus which were separated from a mixture of the viruses and extract from *P. americana* regained their infectivity, and Grasso (6) also showed that southern bean mosaic virus which had separated from its complex with an antiviral protein of *P. americana* partially regained its infectivity. These results indicate that the virus conjugates with the antiviral proteins in the mixture during the local lesion assay and indicate that the conjugation has some effects on virus infectivity. The level of the contribution of the conjugation on the virus inactivation could not be estimated clearly because the strength of the conjugation and the ratio of the antiviral protein to virus was not clear. In the present experiments, we examined the condition for the dissociation of the conjugate between TMV and antiviral protein of *P. americana* and for the adsorption of the antiviral proteins to the cell wall of *Nicotiana tabacum* L. We also examined infectivity in these conditions to estimate the effects of the interactions on TMV inactivation.

MATERIALS AND METHODS

P. americana plants were grown in the field in the summer. PAP and PAPII were purified by a modification of the method of Irvin et al (9,10). Protein extraction was done in a cold room at 4 C. *Phytolacca* leaves, 500 g, were ground with a meat grinder, and the sap was collected and mixed with an equal volume of 60 mM potassium phosphate buffer of pH 6.0. This mixture was filtered through two layers of cheesecloth and the filtrate was centrifuged (15,000 *g* for 15 min at 4 C). To the supernatant, $(NH_4)_2SO_4$ was added to 45% saturation and centrifuged (15,000 *g* for 15 min at 4 C). Then, the 90% $(NH_4)_2SO_4$ saturated fraction of the supernatant was retained and dialyzed against 30 mM phosphate buffer (pH 6.0) for 1 day. DEAE-Sephadex powder was added to the dialysate and it was stirred slowly for 30 min. The fraction not adsorbed on DEAE-Sephadex was loaded onto a CM-Sepharose CL-6B column which had been equilibrated with 30 mM potassium phosphate buffer (pH 6.0), and the proteins were eluted with a 0–500 mM KCl gradient in the same buffer. Two fractions that had inhibitory activity in the TMV local lesion test were collected. The active fractions were concentrated individually and loaded onto a Sephadex G-75 superfine column previously equilibrated with 30 mM potassium phosphate buffer (pH 6.0) and eluted with the same buffer. The fractions having the TMV inhibitory activity were collected and dialyzed against 1 mM phosphate buffer (pH 6.0) for 1 day. Proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis according to Laemmli (12); and the two antiviral proteins were identified by their molecular weights according to Irvin et al (10): 29,000 for PAP and 30,000 for PAPII. Protein concentration was determined from absorbance at 280 nm using the extinction coefficients $E_{1\%} = 8.3$ for PAP and $= 9.1$ for PAPII according to Irvin et al (10). Absorbance was measured with a spectrometer (UV-240, Shimadzu Corp., Kyoto, Japan).

Fluorescein isothiocyanate (FITC)-labeled antiviral proteins were prepared according to Smith et al (19). One ml of 500 mM $NaHCO_3$-Na_2CO_3 buffer (pH 9.4), 0.25 mg of FITC, and 5 mg/10 ml of PAP or PAPII solution were mixed and incubated at 20 C for 10 hr. The mixture was loaded onto a Sephadex G-25 column, and FITC-bound protein was separated. The FITC/protein ratios were calculated to be 0.85 for FITC/PAP and 1.06 for FITC/PAPII from the absorbance values at 280 nm and 495 nm, respectively.

The TMV of the Japanese common strain OM was propagated in *N. tabacum* 'Xanthi' in a greenhouse at 25 ± 1 C. Ten to 14 days after TMV inoculation, the leaves were homogenized and TMV was purified by centrifugation. The homogenate was centrifuged at 10,000 *g* for 15 min at 4 C, and then the supernatant was centrifuged at 95,000 *g* for 1 hr at 4 C. These centrifugations were repeated three times. After the last 95,000-*g* centrifugation, the precipitate was dissolved in 0.5 ml of H_2O, layered on 7 ml of a 10–50% (w/v) sucrose density gradient, and centrifuged at 95,000 *g* for 1 hr at 4 C. An opalescent main band was collected as the TMV solution. TMV concentration was measured from the absorbance of the solution at 260 nm with a spectrometer.

An antiviral protein-TMV complex was made by mixing the protein and TMV. A solution of 0.5 ml of FITC-labeled antiviral protein, 0.5 ml of TMV solution, and 7 ml of H_2O were mixed in an 8-ml centrifuge tube and incubated for 30 min at 25 C to form a TMV-antiviral protein complex. The tube was centrifuged at 95,000 *g* for 1 hr at 4 C. Because pH affects the fluorescence intensity, 2 ml of 50 mM potassium citrate buffer (pH 6.0) containing 500 mM KCl was added to the precipitate and stirred. The fluorescence intensity was measured with a fluorometer (MFP-2A, Hitachi, Ltd., Tokyo, Japan) and the ratio of the antiviral protein to TMV in the precipitate was calculated. To examine the effect of KCl on the antiviral protein-to-TMV ratio, the KCl concentration in the protein-TMV mixture was adjusted from 0 to 100 mM. To examine the effect of pH on the antiviral protein-to-TMV ratio, the pH of the protein-TMV mixture was adjusted from 2 to 12 with HCl or NaOH solution. The pH of the mixture was measured with a small pH electrode (L-7LC, Horiba, Ltd., Kyoto, Japan).

Adsorption of the FITC-labeled antiviral protein to plant cells was observed by fluorescence microscopy (Fluophot, Nihon Kougaku, Ltd., Tokyo, Japan). A white cell strain of *N. tabacum* 'Xanthi' cultured in Murashige and Skoog's medium (13) containing 10^{-5} M 2,4-dichlorophenoxyacetic acid was used for this experiment. Cells were washed with 1 mM potassium phosphate buffer (pH 6.0) and suspended in the diluted FITC-labeled antiviral protein solution. The pH of the FITC-labeled protein solution was adjusted with 25 mM buffers (pH 3.0 and 5.5: sodium citrate; pH 8.0: tris(hydroxymethyl)aminomethane-HCl).

The local lesion assay was performed on 2- to 3-mo-old plants of *N. glutinosa* in a greenhouse controlled at 25 ± 2 C. The fourth to eighth leaves from the top of the plant were used. Carborundum of 600 mesh was sprinkled on the tobacco leaves and TMV solution or a TMV-antiviral protein mixture was rubbed on the adaxial leaf surface with a small cotton ball. Local lesions on the leaf surface were counted after 4 days.

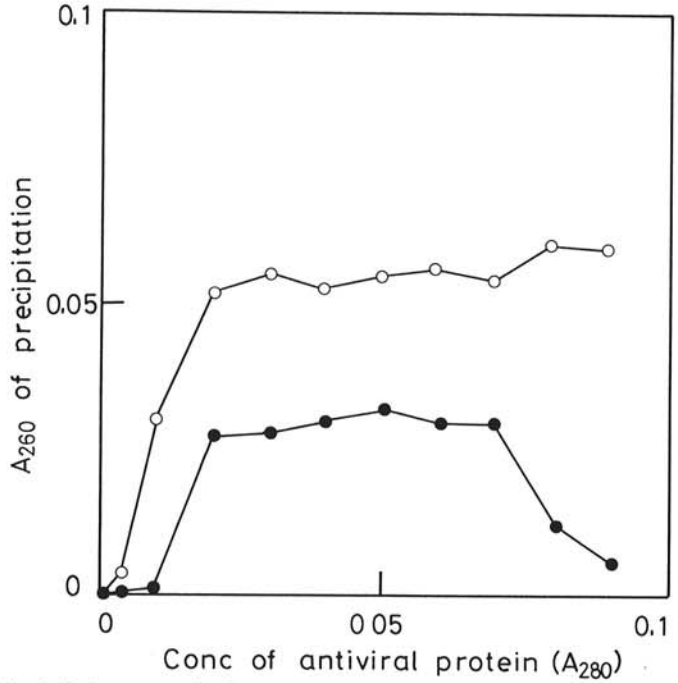

Fig. 1. Tobacco mosaic virus (TMV) precipitation by pokeweed antiviral proteins (PAP). A mixture of 0–3.7 × 10^{-6} M PAP or 0–3.3 × 10^{-6} M PAPII (A_{280nm} = 0–0.09) and 8.5 × 10^{-10} M (A_{260mn} = 0.1) TMV was centrifuged at 5,000 *g* for 15 min. The TMV-antiviral protein precipitate was dissolved with 2 ml of 500 mM KCl solution and the absorbance at 260 nm was measured. Open circles indicate the absorbance values of TMV-PAP precipitations; closed circles indicate the absorbance values of TMV-PAPII precipitations.

RESULTS

TMV precipitation by antiviral proteins. Samples of PAP or PAPII (A_{280nm} = 0.1/PAP, 4.2 × 10^{-6} M, PAPII, 3.7 × 10^{-6} M; 0–0.9 ml), TMV (A_{260nm} = 1.0/8.5 × 10^{-10} M, 0.1 ml), and H_2O (0–0.9 ml) were mixed in glass tubes. When 0.2–0.9 ml of these proteins were used, a white precipitate was observed and

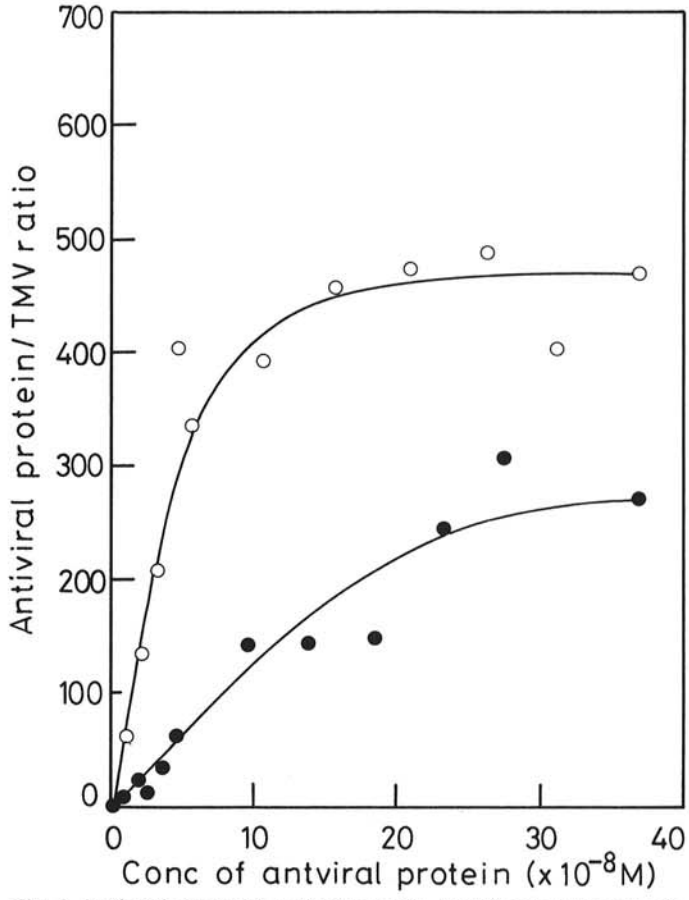

Fig. 2. Ratio of pokeweed antiviral protein (PAP) to tobacco mosaic virus (TMV) in the TMV-protein complex. Fluorescein isothiocyanate (FITC)-labeled PAP or PAPII was mixed with TMV in an 8-ml centrifuge tube. TMV, PAP, and PAPII concentrations were 1.1 × 10^{-10} M, 1.1 × 10^{-8} to 3.7 × 10^{-7} M, and 9.5 × 10^{-9} to 3.7 × 10^{-7} M, respectively. After centrifugation (95,000 *g* for 1 hr at 4 C), the precipitate was dissolved in 2 ml of 50 mM potassium citrate buffer (pH 6.0) containing 500 mM KCl, and the fluorescence intensity was measured by fluorometry. The number of antiviral proteins conjugated with one molecule of TMV was calculated from the fluorescence intensity. Open circles indicate PAP/TMV ratios; closed circles indicate PAPII/TMV ratios.

the mixture became turbid. The precipitate was collected by centrifugation at 5,000 *g* for 15 min and dissolved in 2 ml of 500 mM KCl solution to dissociate the complex, and the absorbance of the solution at 260 nm was measured. Most of the TMV could be precipitated by the addition of PAP ($A_{280nm} = 0.02$–0.09/ 8.4×10^{-7} to 3.7×10^{-6} M), and about half by addition of PAPII ($A_{280nm} = 0.02$–$0.07/7.4 \times 10^{-7}$ to 2.6×10^{-6} M), as shown in Figure. 1. When the concentration of PAPII was more than 3.0×10^{-6} M, the quantity of the precipitate decreased.

Ratio of antiviral protein/TMV. TMV was mixed with various concentrations of FITC-labeled PAP or PAPII in individual centrifuge tubes. In this experiment, no white precipitate was observed, and the precipitate could not be collected by centrifugation at 5,000 *g*. TMV, PAP, and PAPII concentrations in the tube were 1.1×10^{-10} M, 1.1×10^{-8} to 3.7×10^{-7} M, and 9.5×10^{-9} to 3.7×10^{-7} M, respectively. All of the TMV was precipitated by ultracentrifugation (95,000 *g* for 1 hr at 4 C), and the number of antiviral proteins attached to TMV molecules was calculated from the fluorescence intensity of the precipitate. The molecular weight of TMV is 39,000,000, according to Boedtker and Simmons (3). In proportion to an increase in the concentration of the antiviral proteins added, the TMV/antiviral protein ratio increased (Fig. 2). When the concentration of PAP in the mixture was 1.6×10^{-7} to 3.7×10^{-7} M, the PAP/TMV ratio reached about 450:1; and when the mixed PAPII was 3.0×10^{-7} M, the PAPII/TMV ratio reached about 300:1.

To examine the dissociation of the TMV-antiviral protein complex by KCl, PAP or PAPII was mixed with TMV in 0–100 mM KCl solutions. TMV, PAP, and PAPII concentrations in the centrifuge tube were 1.1×10^{-10} M, 5.8×10^{-8} M, and 2.3×10^{-7} M, respectively. Most of the PAP and PAPII was dissociated in 20–100 and 60–100 mM KCl solution, respectively (Fig. 3). The ratio probably did not become zero in 80–100 mM KCl because the supernatant in the centrifuged tube could not be completely removed.

The effect of pH on the antiviral protein/TMV ratio is shown in Figure. 4. TMV, PAP, and PAPII concentrations in the centrifuge tube were 1.1×10^{-10} M, 2.6×10^{-7} M, and 2.3×10^{-7} M, respectively. Antiviral proteins binding to TMV showed a bell-shaped curve. The maximum PAP/TMV ratio was 700:1 at pH 5.0 and the maximum PAPII/TMV ratio was 440:1 at pH 6.0.

Local lesion test. Inhibition of TMV infectivity by pokeweed antiviral proteins was examined. TMV solution was rubbed on the left surface of the *N. glutinosa* leaf and TMV-antiviral protein mixture was rubbed on the right surface of the same leaf. The concentration of TMV was 2.3×10^{-9} M or 2.6×10^{-10} M, that of PAP was 4.2×10^{-11} to 4.2×10^{-7} M, and that of PAPII was 3.7×10^{-11} to 3.7×10^{-7} M. Local lesions were counted after 4 days (Table 1). When the TMV concentration was 2.3×10^{-9} M or 2.6×10^{-10} M, 4.2×10^{-7} M PAP and 3.7×10^{-7} M PAPII completely inhibited the expression of local lesions, 4.2×10^{-8} M PAP and 3.7×10^{-8} M PAPII inhibited it by about 90–100%, and 4.2×10^{-9} M PAP and 3.7×10^{-9} M PAPII inhibited it by more than 60%. Thus, the inhibitory effect depended on the concentration of the antiviral protein. When the TMV

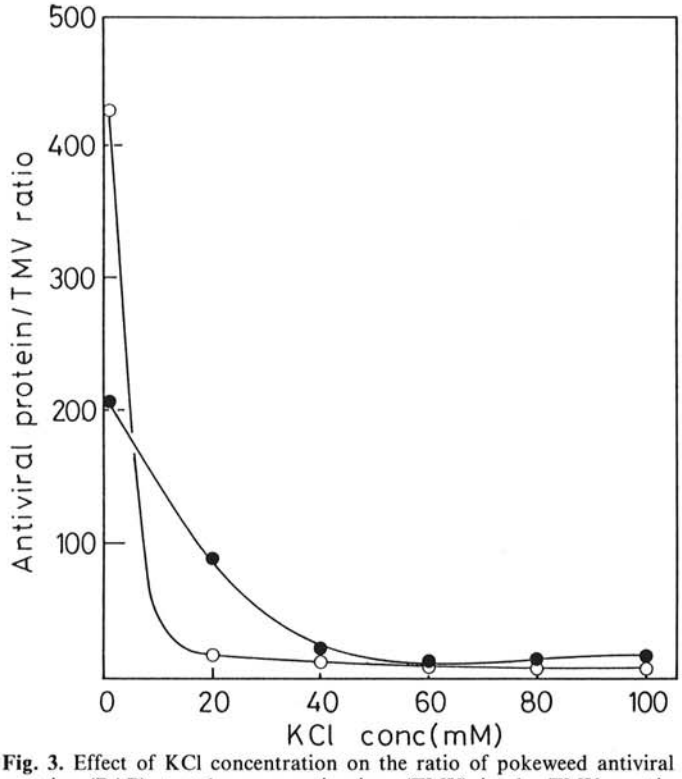

Fig. 3. Effect of KCl concentration on the ratio of pokeweed antiviral proteins (PAP) to tobacco mosaic virus (TMV) in the TMV-protein complex. Fluorescein isothiocyanate (FITC)-labeled PAP or PAPII was mixed with TMV in 0–100 mM KCl solution. TMV, PAP, and PAPII concentrations in the mixture were 1.1×10^{-10} M, 5.8×10^{-8} M, and 2.3×10^{-7} M, respectively. The mixture was centrifuged (95,000 *g* for 1 hr at 4 C). The precipitate was dissolved in 2 ml of 50 mM potassium citrate buffer (pH 6.0) containing 500 mM KCl, and the fluorescence intensity was measured by fluorometry. The number of antiviral proteins conjugated with one molecule of TMV was calculated from the fluorescence intensity. Open circles indicate PAP/TMV ratios; closed circles indicate PAPII/TMV ratios.

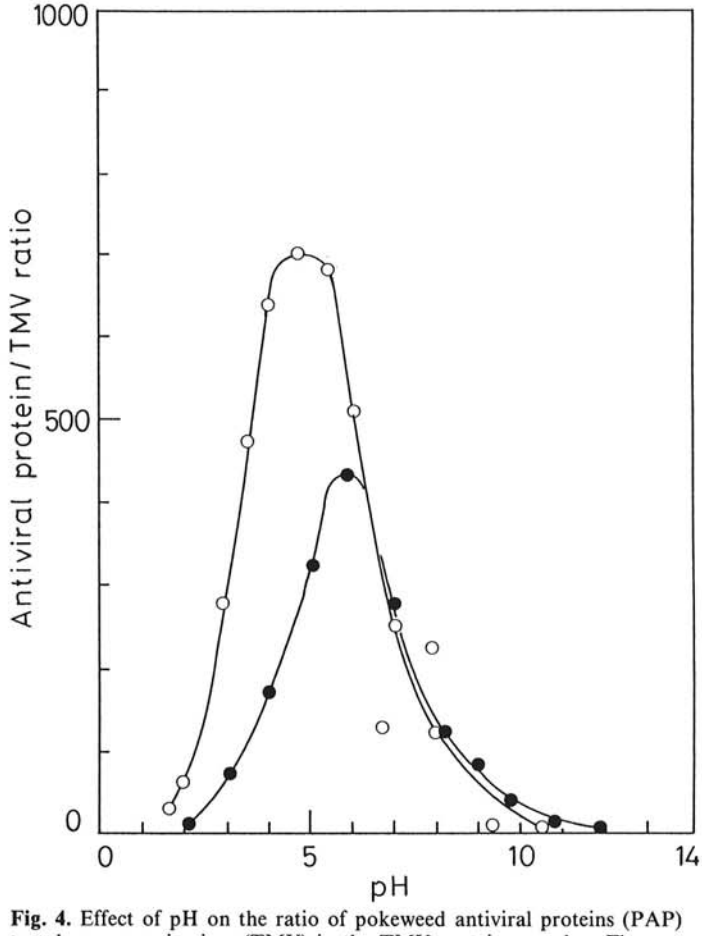

Fig. 4. Effect of pH on the ratio of pokeweed antiviral proteins (PAP) to tobacco mosaic virus (TMV) in the TMV-protein complex. Fluorescein isothiocyanate (FITC)-labeled PAP or PAPII was mixed with TMV and the pH of the mixture was adjusted to 2–12 with HCl or NaOH solution. TMV, PAP, and PAPII concentrations in the mixture were 1.1×10^{-10} M, 2.6×10^{-7} M, and 2.3×10^{-7} M, respectively. The mixture was centrifuged (95,000 *g* for 1 hr at 4 C). The precipitate was dissolved in 2 ml of 50 mM potassium citrate buffer (pH 6.0) containing 500 mM KCl, and the fluorescence intensity was measured by fluorometry. The number of antiviral proteins conjugated with one molecule of TMV was calculated from the fluorescence intensity. Open circles indicate PAP/TMV ratios; closed circles indicate PAPII/TMV ratios.

TABLE 1. Effect of pokeweed antiviral proteins (PAP and PAPII) on the infectivity of tobacco mosaic virus (TMV)

Antiviral protein	Concentration of antiviral protein (M)	Inhibition (%)	
		Concentration of TMV 2.3×10^{-9} M	Concentration of TMV 2.6×10^{-10} M
PAP	4.2×10^{-7}	100.00	100.00
	4.2×10^{-8}	100.00	100.00
	4.2×10^{-9}	98.90	80.10
	4.2×10^{-10}	6.00	35.10
	4.2×10^{-11}	1.20	10.80
PAPII	3.7×10^{-7}	100.00	100.00
	3.7×10^{-8}	97.90	100.00
	3.7×10^{-9}	77.10	82.20
	3.7×10^{-10}	28.50	61.90
	3.7×10^{-11}	22.50	41.30

TMV solution was rubbed on the left adaxial surface of a leaf of *Nicotiana glutinosa*, and a TMV and antiviral protein mixture was rubbed on the right surface of the same leaf. Local lesions on the leaves were counted after 4 days and the inhibition rate (%) was calculated with the following equation:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{local lesions on the right surface}}{\text{local lesions on the left surface}}\right) \times 100$$

TABLE 2. Local lesions caused by tobacco mosaic virus (TMV) dissociated from the complex of TMV and pokeweed antiviral proteins (PAP and PAPII)[a]

Concentration of antiviral protein and TMV	Sample	Local lesions per leaf, mean ± S.D.[b]
PAP (0 M) TMV (1.3×10^{-9} M)	$Control_1$	555.6 ± 102.1
PAP (0 M) TMV (1.2×10^{-8} M)	$Control_2$	714.7 ± 163.5
PAP (4.2×10^{-6} M) TMV (2.6×10^{-9} M)	1	0.0 ± 0.0
	2	47.5 ± 27.2
PAP (4.2×10^{-6} M) TMV (2.3×10^{-8} M)	1	0.0 ± 0.0
	2	321.0 ± 65.0
PAPII (3.7×10^{-6} M) TMV (2.6×10^{-9} M)	1	0.0 ± 0.0
	2	190.3 ± 36.4
PAPII (3.7×10^{-6} M) TMV (2.3×10^{-8} M)	1	0.0 ± 0.0
	2	173.5 ± 111.5

[a] A mixture of 0.5 ml of 4.2×10^{-6} M PAP or 3.7×10^{-6} M PAPII with 0.5 ml of 2.6×10^{-9} M or 2.3×10^{-8} M TMV was centrifuged at 5,000 *g* for 15 min at 25 C. The precipitate was dissolved in 1 ml of H_2O and separated into two 0.5-ml fractions. To one fraction, 0.5 ml of H_2O was added and the solution was used as sample 1. To the other fraction, 7.5 ml of 500 mM KCl was added and the mixture was centrifuged at 95,000 *g* for 1 hr at 4 C. The precipitate was dissolved with 8 ml of 500 mM KCl solution and centrifuged at 95,000 *g*. The precipitate was dissolved with 1 ml of H_2O and used as dissociated TMV sample 2. Samples 1 and 2 were rubbed on the leaf surface of *Nicotiana glutinosa*, and the number of local lesions was counted after 4 days. $Control_1$ and $control_2$ are samples of only TMV.

[b] S.D. is the standard deviation of three determinations.

concentration was higher than that of the antiviral protein, partial TMV inactivation was observed; that is, when 4.2×10^{-11} M PAP or 3.7×10^{-11} M PAPII was mixed with 2.3×10^{-9} M TMV, the inhibition rate was 1.2 and 22.5%, respectively.

The infectivity of the dissociated TMV from the TMV-antiviral protein complex is shown in Table 2. In this experiment, 0.5 ml of 4.2×10^{-6} M PAP or 3.7×10^{-6} M PAPII was mixed with 0.5 ml of 2.6×10^{-9} M TMV or 2.3×10^{-8} M TMV in an Eppendorf tube and incubated for 30 min at 25 C. The mixture was centrifuged at 5,000 *g* for 15 min at 25 C. The precipitate of the TMV-antiviral protein complex was dissolved in 1 ml of H_2O and the solution was divided into 0.5-ml fractions. To one fraction, 0.5 ml of H_2O was added and the solution was used as sample 1. To the other, 7.5 ml of 500 mM KCl was added, the TMV-antiviral protein complex was dissociated, and the solution was centrifuged at 95,000 *g* for 1 hr. The precipitate was dissolved in 7.5 ml of 500 mM KCl and centrifuged at 95,000 *g*. This precipitate was dissolved with 1 ml H_2O and the redissociated TMV was used as sample 2. Samples 1 and 2 were rubbed on separate leaf surfaces of *N. glutinosa*. Leaves rubbed with sample 1 showed no local lesions, but those rubbed with sample 2 did (Table 2). This shows that the TMV dissociated from the TMV-antiviral protein complex regained its infectivity.

To test the effect of KCl on the local lesion test, 8.5×10^{-10} M TMV solution containing 0, 100, 200, or 300 mM KCl was inoculated on leaves of *N. glutinosa*. These treatments had little effect on numbers of local lesions (Table 3). When 8.5×10^{-10} M TMV solution containing 4.2×10^{-7} M PAP or 3.7×10^{-7} M PAPII in 0, 100, 200, and 300 mM KCl was rubbed on the leaves of *N. glutinosa*, no local lesions were observed and TMV infectivity was not recovered.

Adsorption of PAP on the cell wall. When a strain of cultured white cells of *N. tabacum* 'Xanthi' was suspended in FITC-labeled PAP solution, high fluorescence was observed in the cells, showing that FITC-labeled PAP became adsorbed on the cells. PAP adsorption was observed at pH. 3.0, 5.5, and 8.0 (Fig. 5A–C). It occurred in 200 mM KCl (Fig. 5D), but not in 300 mM KCl.

DISCUSSION

We examined the precipitation of TMV with antiviral proteins of *P. americana* quantitatively and determined conditions for dissociation. The maximum ratio of PAP to TMV in the precipitate obtained by 95,000 *g* centrifugation was 700:1 at pH 5 and that for PAPII/TMV was 440:1 at pH 6. These ratios were below 100 at pH 2 and pH 9–12, respectively. These conjugates were dissociated perfectly in over 60 mM KCl solution. These results suggest that the conjugation involves a weak ionic bond. Our results resemble those of Kassanis and Kleczkowski (11): The precipitate of TMV and an antiviral protein of *P. esculenta* is induced by addition of 1% NaCl or changing the pH to below 3 or above 7. When TMV (8.5×10^{-10} M) was mixed with PAP (3.7×10^{-7} to 3.7×10^{-6} M) or PAPII (3.3×10^{-7} to 3.3×10^{-6} M), the mixture was turbid and most of TMV was precipitated at low-speed centrifugation (5,000 *g*) (Fig. 1). It is likely that

TABLE 3. Effect of pokeweed antiviral proteins (PAP and PAPII) on the infectivity of tobacco mosaic virus (TMV) in 0, 100, 200, and 300 mM KCl

KCl concentration (mM)	Number of local lesions per leaf (number ± S.D.[a])		
	TMV[b] (8.5×10^{-10} M)	TMV[c] (8.5×10^{-10} M) + PAP (4.2×10^{-7} M)	TMV[d] (8.5×10^{-10} M) + PAPII (3.7×10^{-7} M)
0	473.8 ± 145.6	0.0 ± 0.0	0.0 ± 0.0
100	359.3 ± 15.6	0.0 ± 0.0	0.0 ± 0.0
200	234.3 ± 81.3	0.0 ± 0.0	0.0 ± 0.0
300	393.5 ± 31.7	0.0 ± 0.0	0.0 ± 0.0

[a] S.D. is the standard deviation of five determinations.

[b] TMV solutions containing 0–300 mM KCl were rubbed on the leaf surface of *Nicotiana glutinosa*.

[c] TMV solutions containing 0–300 mM KCl and PAP were rubbed on the leaf surface of *N. glutinosa*.

[d] TMV solutions containing 0–300 mM KCl and PAPII were rubbed on the leaf surface of *N. glutinosa*. Local lesions on the leaves were counted after 4 days.

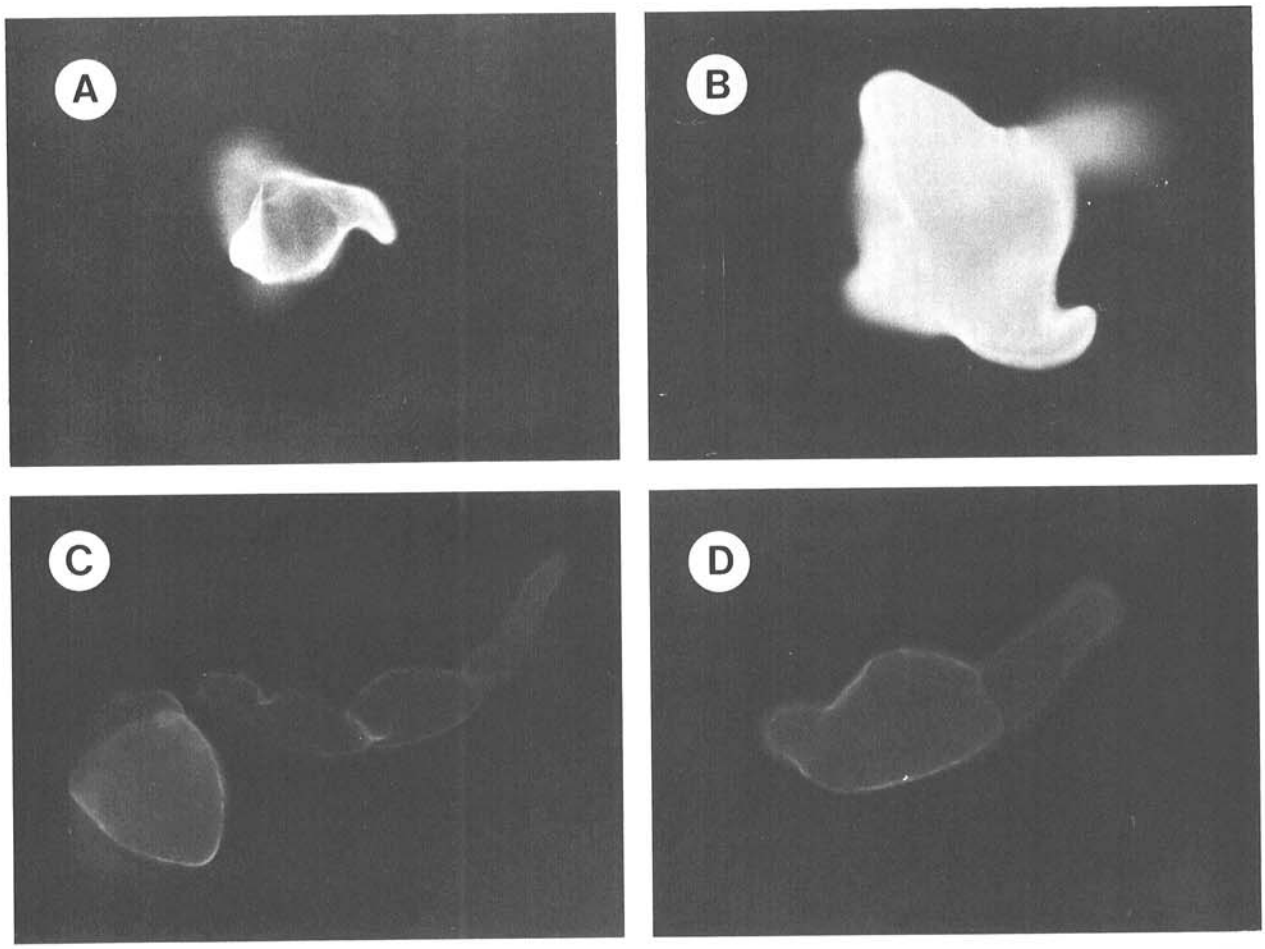

Fig. 5. Adsorption of pokeweed antiviral protein (PAP) to the cell wall of cultured cells of *Nicotiana tabacum* 'Xanthi'. White cells lacking chloroplasts were suspended in the solution containing fluroescein isothiocyanate (FITC)-labeled protein and the cells were observed by fluorescence microscopy. **A,** A cell suspended in 25 mM sodium citrate buffer (pH 3.0) containing FITC-labeled PAP. **B,** A cell suspended in 25 mM sodium citrate buffer (pH 5.5) containing FITC-labeled PAP. **C,** Cells suspended in 25 mM tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0) containing FITC-labeled PAP. **D,** Cells suspended in 200 mM KCl solution containing FITC-labeled PAP. Magnification of all photographs , × 175.

when the local lesion assay was done using the mixture of TMV and antiviral proteins, TMV was retained on the leaf surface as a precipitate. Infectivity of TMV must be inhibited partially by the conjugation.

We found that the formation of local lesions was completely inhibited by PAP and PAPII when mixtures of TMV, antiviral protein, and 0–300 mM KCl were inoculated on the leaves of *N. glutinosa* (Table 3). The conjugation of TMV and antiviral protein is dissociated completely at 300 mM KCl; therefore, conjugation is not the main cause for the inhibition of TMV.

When the mixture of TMV and antiviral protein without KCl was inoculated on the leaves of *N. glutinosa*, TMV inhibition occurred, even if the TMV concentration was higher than that of the antiviral proteins (Table 1). The pokeweed antiviral proteins could completely inhibit TMV infection at only 0.01 mg/ml. Stahmann et al (21) reported that 0.4 mg/ml of lysine basic polypeptide is needed for inhibition of the TMV infection. These results suggest that TMV infection is not fully inhibited by the conjugation with antiviral protein, but by some other effects of antiviral proteins on the host cell. The reason why the complex of TMV-antiviral protein had no infectivity (Table 2) may involve the effects of the antiviral proteins which are around TMV on the host cell. Inhibitory effect of an antiviral protein of *P. americana* on in vitro protein synthesis using ribosomes of *N. tabacum* is reported by Battelli et al (2). However, there are no data that antiviral proteins or the complex of TMV and the antiviral proteins invaded the cell.

PAP adsorption to the cultured cell of *N. tabacum* was found in 0–200 mM KCl solution. PAPII also became adsorbed into the tobacco cell (data not shown). Because no adsorption of PAP to protoplast was observed, this adsorption may be due to the basic protein PAP conjugating with cell wall components. The adsorption of a basic protein or an antiviral protein onto plant tissue has been reported by previous workers: Show (20) found that poly-L-ornithine promotes retention of TMV on tobacco leaves and the uncoating of the virus. Ready et al (18) found by electron microscopy that an antiviral protein of *P. americana* bound with the cell wall matrix of leaf mesophyll cells of the plant.

In local lesion assays, when a basic antiviral protein and TMV solution were jointly inoculated onto the tobacco leaf surface, the cell wall of the injured part on the leaf may be a site for adsorption by the protein resulting in neutralization of the charge of cell.

From these experiments, we concluded that the strength of the conjugation of TMV and antiviral protein (PAP and PAPII) and the adsorption of antiviral protein to cell walls is too weak to fully inhibit TMV infectivity and these interactions are not necessary conditions for TMV inactivation. The interactions may have some minor role for TMV inactivation in low-ionic-strength conditions. It is likely that TMV infection is inhibited partially by the precipitate of most of the TMV by the antiviral protein.

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