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Full Length Research Paper

Spinacia oleracea proteins with antiviral activity against *tobacco mosaic virus*

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Tobacco mosaic virus **(TMV) is a devastating microorganism with a global distribution and a wide host range. Protein extracts isolated from *Spinacia oleracea* (spinach) were examined with bioassay-guide and tested for the resistance to TMV in the *Nicotiana glutinosa* leaves by local lesion assay. The results show that the crude extract and the fraction of 0 to 40% ammonium sulfate precipitation could protect tobacco from TMV infection. An active fraction was further purified with cation-exchange chromatography (SP-sepharose HP) and activity screening. The inhibitory rate for TMV *in vitro* was up to 94.35% with 50 µg/mL of the active fraction. In addition, this active fraction could function against TMV at the infection sites on leaves of *N. glutinosa*. However, it had only a little inhibitory effect on TMV replication and the mechanism under the TMV-inhibitory activity of protein extract from spinach still need further investigation.**

Key words: *Tobacco mosaic virus***,** spinach, *Nicotiana glutinosa*, chromatography, active fraction.

INTRODUCTION

Tobacco virus diseases are a class of widespread diseases on tobacco plants and destructive to tobacco yield and quality. Till now, 27 viruses have been isolated from tobacco, including *Tobacco mosaic virus* (TMV), *Cucumber mosaic virus* (CMV), *Potato virus* Y (PVY), *Tobacco etch virus* (TEV), *Tobacco ring spot virus* (TRSV), etc. Once infected by tobacco viruses, tobacco inherent chemical composition becomes unbalanced, leaf qualities deteriorate, yields and economic benefits decline dramatically (Lei, 2004). While inhibiting the viruses, anti-viral drugs may be harmful to the host plants, which are a major problem in controlling of plant virus diseases (Wu et al., 2008).

TMV belongs to and is one of the representatives of the *tobamovirus* genus, which distribute widely all over the world and cause extensive damage for crop plants (Dawson et al., 1986). TMV genome is composed of a single strand, positive strand RNA about 6.3 to 6.6 kb in length (Goelet et al., 1982). It could infect more than 300 plant species, especially detrimental for tobacco plants. The most appropriate temperature for TMV proliferation is about 28 to 30°C. However, the virus is extremely stable *in vitro*. The virus could even live for more than 50 years in the dry tissues of plant (Zaitlin and Israel, 1975). Therefore, TMV prevention is a major problem for the tobacco production in the fields. The main measures in TMV defense include the using of TMV-resistant varieties, systematic cultivation techniques, chemically antiviral agents. Since the first plant virus resistant protein pokeweed antiviral proteins (PAP) was isolated from *Phytolacca esculenta* (Kassanis et al., 1948), scientists made great attempts at finding out the antiviral proteins from other plants. Nevertheless, a small number of plant anti-TMV protein extracts were obtained from *Basella rubra*, *Bougainvillea spectabilis* (Bolognesi et al., 2003), *Boerhavia diffusa*, (Verma et al., 1979), *Bougainvillea xbuttiana* (Narwal et al., 2001), *Bryonia dioica* (Stirpe et al., 1981), Cetosia cristata (Balasubrahmanyama et al., 2000), *Chenopodium amaranticolor* (Taniguchi and Goto, 1976), *Clerodendrum aculeatum* (Verma et al., 1996), *Clerodendrum inerme* (Olivieri et al., 1996), *Cuscuta*

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Abbreviation: TMV, Tobacco mosaic virus;

reflexa (Awasthi et al., 1981), *Dinathus barbarus* (Taniguchi and Goto, 1980), *Dinathus caryophyllus* (Stripe et al., 1981), *Mirabilis jalapa* (Kubo et al., 1990), *Momordica charantia* (Stevens et al., 1981), *Nicotiana tabacum* (Edelbaum et al., 1990), *Phytolacca americana* (Irvin et al., 1980; Bolognesis et al., 1990), *Sambucus nigra* (Chen et al., 2002), *Silene schafta* (Alexandre et al., 1997), *Yucca recurvifolia salisb* (Naofumi and Akira, 1987), etc.

The annual or biennial spinach belongs to the Chenopodiaceae family and is widely cultivated in China. Currently, little has been reported about the application of spinach proteins in TMV control except that spinach crude extract could protect *Nicotiana glutinosa* plants from TMV (Cho et al., 2000). In this research, we further separated the anti-TMV proteins from the spinach crude extract. The mechanism under the anti-TMV ability of the active protein components was also primarily investigated.

MATERIALS AND METHODS

Materials

N. glutinosa plants were used as local lesion host for bioassay and were grown in a growth chamber under 16 h light/8 h dark cycles at 25°C, with 70% humidity. Uniform plants were selected for experiments. TMV was used to test the virus inhibitory activities of the spinach leaf extracts. The virus was maintained on its systemic host, K_{326}. Spinach plants were purchased from local market.

Purification of TMV

TMV was purified according to Gooding (Gooding and Hebert, 1967).

Extraction and primary isolation of spinach protein

Spinach leaves were homogenized with three volumes of 0.15 mol/L phosphate buffer (pH7.2), containing 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L ethylenediaminetetraacetic acid (EDTA) and 0.5 mmol/L *dithiothreitol* (DTT). The homogenate was squeezed through two layers of muslin cloth, and filtrate was centrifuged at 10000 g for 10 min. The supernatant was used for primary purification.

The supernatant obtained from leaf extract was subjected to ammonium sulfate fractionation at 40% saturation, by slow addition of solid ammonium sulfate with constant stirring at 4°C. The precipitated protein was centrifuged at 10000 g for 20 min. The precipitated protein pellet was suspended in minimal amount of 0.01 mol/L phosphate buffer (pH 6.2) and dialyzed overnight against the same buffer at 4°C.

For further purification, chromatography on SP Sepharose column was used. After equilibrating the SP Sepharose column with several volumes of 0.01 mol/L phosphate buffer (pH6.2), the active 0 to 40% ammonium sulfate dialyzed protein fraction was loaded on the column and washed with equilibration buffer. The adsorbed protein was then eluted with a stepwise sodium chloride gradients (0.1, 0.25 and 0.5 mol/L), which was prepared in equilibration buffer. The eluted components were collected using a fraction collector system, at a flow rate of 1 mL/min. The eluted components were simultaneously monitored for absorbance at 280 nm. The protein fractions were separately pooled and tested for the antiviral activity.

Protein samples were analyzed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide gel and visualized by staining with Coomassie Brilliant Blue R250 (Laemmli et al., 1970).

Estimation of antiviral activity

The antiviral activity of 0 to 40% ammonium sulfate fraction was estimated by local lesion assay. 0 to 40% ammonium sulfate fraction was mixed with equal volume of TMV (15 µg/mL) in 0.01 mol/L phosphate buffer (pH 6.2) and the mixture was inoculated on carborundum-dusted *N. glutinosa* half-leaves. Half-leaves were rubbed with TMV solution that had been mixed with phosphate buffer as control. For each treatment, three plants with three to four leaves of equal size were used. Tobacco plants were observed for the development of lesions for three days. The antiviral activity of the proteins was calculated in terms of percentage inhibition using the following formula: inhibition = (C-T)/ C × 100%, C is the average number of lesions in control plants, and T is the average number of lesions in treated plants.

The inhibitory effect of SPF5 peak-proteins against TMV *in vitro*

N. glutinosa leaves were dusted with carborundum. 25, 12.5, 6.25 and 3.125 µg/mL SPF5 fraction were mixed with equal volume of 15 µg/mL TMV respectively and incubated for 30 min. The mixtures were mechanically inoculated onto one side leaves of *N. glutinosa* as the treatment, while another side of the leaves was inoculated with TMV solution diluted with the same volume of phosphate buffer buffer as the negative control.

The inhibitory effect of SPF5 peak-proteins against TMV at the infection sites

The half leaves of *N. glutinosa* were rubbed with 50 µg/mL SPF5 fraction without carborumdum and another half leaves were rubbed with phosphate buffer as control. All test leaves were inoculated with TMV after 2, 5 and 9 h independently.

The inhibitory activity against TMV replication of SPF5 peak-proteins

Growing leaves of *N. glutinosa* were inoculated both sides with equal volume of TMV. After 2, 5 and 9 h, the right side of the leaves were treated with the SPF5 fraction. The left half leaves were rubbed with phosphate buffer as control.

RESULTS

The antiviral activity of spinach crude extract and 0 to 40% $(NH_4)_2SO_4$ saturated fraction

The antiviral activity of spinach crude extract was examined by local lesion. The results show that the inhibitory rate was 80.75% when 0.232 mg/mL crude extract was used, while the antiviral activity was up to 92.36% when 0.325 mg/mL protein of 0 to 40% $(NH_4)_2SO_4$ saturated fraction was used (Table 1). It was

Table 1. Inhibition of TMV infection by the crude extract and 0 to 40% $(NH_4)_2SO_4$ saturated fraction from spinach.

Protein extract	Sample concentration (µg/mL)	Average lesion number[a]		Inhibitory rate (%)
		Treated	Control	
Crude extract	246	9.31	48.50	80.80
0-40%(NH4)2SO4 saturated fraction	217	3.73	47.90	92.21

[a]Mean number of lesions on 9th to 10th leaves of nine tested plants from three independent experiments. TMV, *Tobacco mosaic virus*.

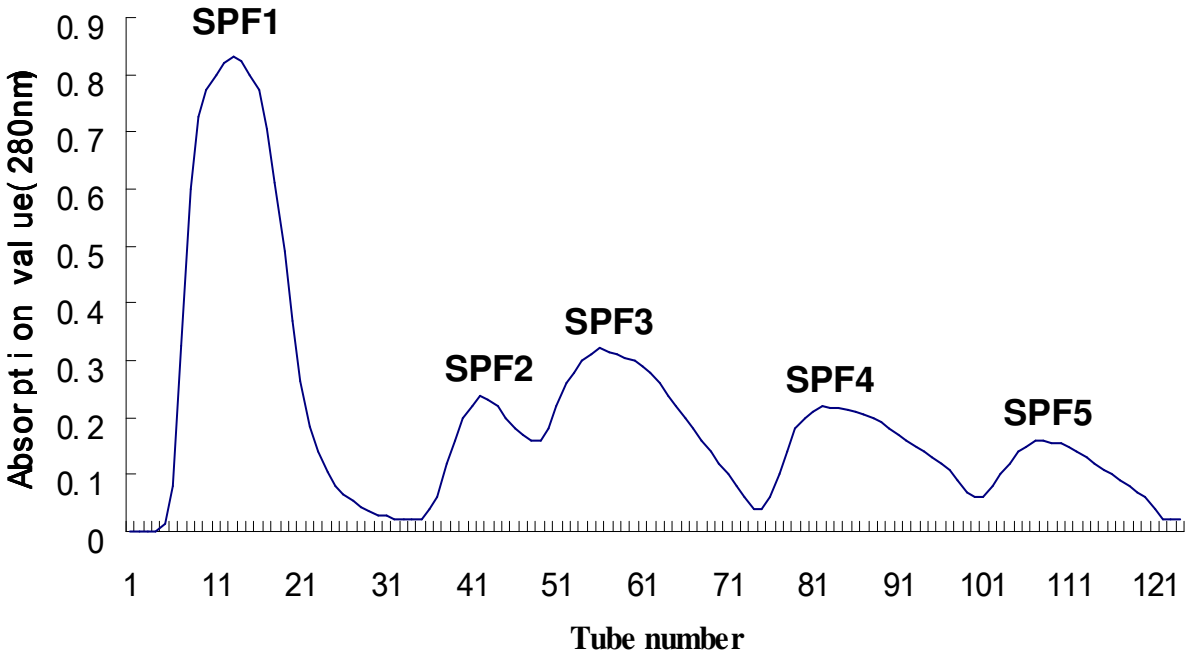

Figure 1. Cation-exchange chromatography of 0 to 40% ammonium sulfate precipitation fractions from spinach on SP-sepharose HP column. The displayed pictures are representative of three independent experiments.

demonstrated that 0 to 40% $(NH_4)_2SO_4$ saturated fraction contained a lot of antiviral proteins.

Chromatography of 0 to 40% $(NH_4)_2SO_4$ saturated fraction on SP Sepharose column

The active fraction from ammonium sulfate fractionation was loaded onto a SP Sepharose column which had been equilibrated with 0.01 mol/L phosphate buffer (pH 6.2). Equilibration buffer was used to wash the non-specific binding proteins, and then a discontinuous gradient of NaCl (0.1, 0.25 and 0.5 mol/L) solutions were used to elute specific binding proteins. Five protein peaks appeared in the elution process (Figure 1). Peak was designated as SPF1 which was washed down by equilibration buffer. Such unabsorbed fraction indicated that the protein had the basic nature. Another two protein peaks were collected and named as SPF2 and SPF3 respectively when the elution buffer containing 0.1 mol/L NaCl was used. Accordingly, SPF4 and SPF5 were obtained separately corresponding to 0.25 mol/L and 0.5 mol/L NaCl elution buffers.

Furthermore, five protein peaks were pooled and dialyzed against ultra-pure water overnight at 4℃. The inhibitory activity of five collected peak-proteins was tested. The results suggested that the inhibitory rates of different peak-proteins (SPF1, SPF2, SPF3, SPF4) were 17.78, 49.81, 43.53 and 32.58% respectively (Table 2). The SPF5 peak-proteins exhibited highly effective antiviral activity (91.43% local lesion inhibition) (Figure 2a), which was consistent with the results got from 0 to 40% $(NH_4)_2SO_4$ saturated fraction, with relatively lower protein concentration compared to other four peak-proteins. The protein components of SFP5 peak-proteins were separated by SDS-PAGE, and there were four main protein bands in the lane (Figure 2b).

The inhibitory effect of SPF5 peak-proteins against TMV *in vitro*

Different concentrations of SPF5 peak-proteins were

Table 2. The inhibitory effect against TMV of protein peaks from spinach purified with cation-exchange chromatography.

Protein peak	Sample concentration (μg/mL)	Average lesion number[a]		Inhibitory rate (%)
		Treated	Control	
SPF1	566.00	33.71	41.00	17.78
SPF2	462.67	22.83	45.50	49.81
SPF3	289.16	24.00	42.50	43.53
SPF4	64.81	29.67	44.00	32.58
SPF5	31.03	3.67	42.78	91.43

[a]Mean number of lesions on 9th to 10th leaves of nine tested plants from three independent experiments. TMV, *Tobacco mosaic virus*.

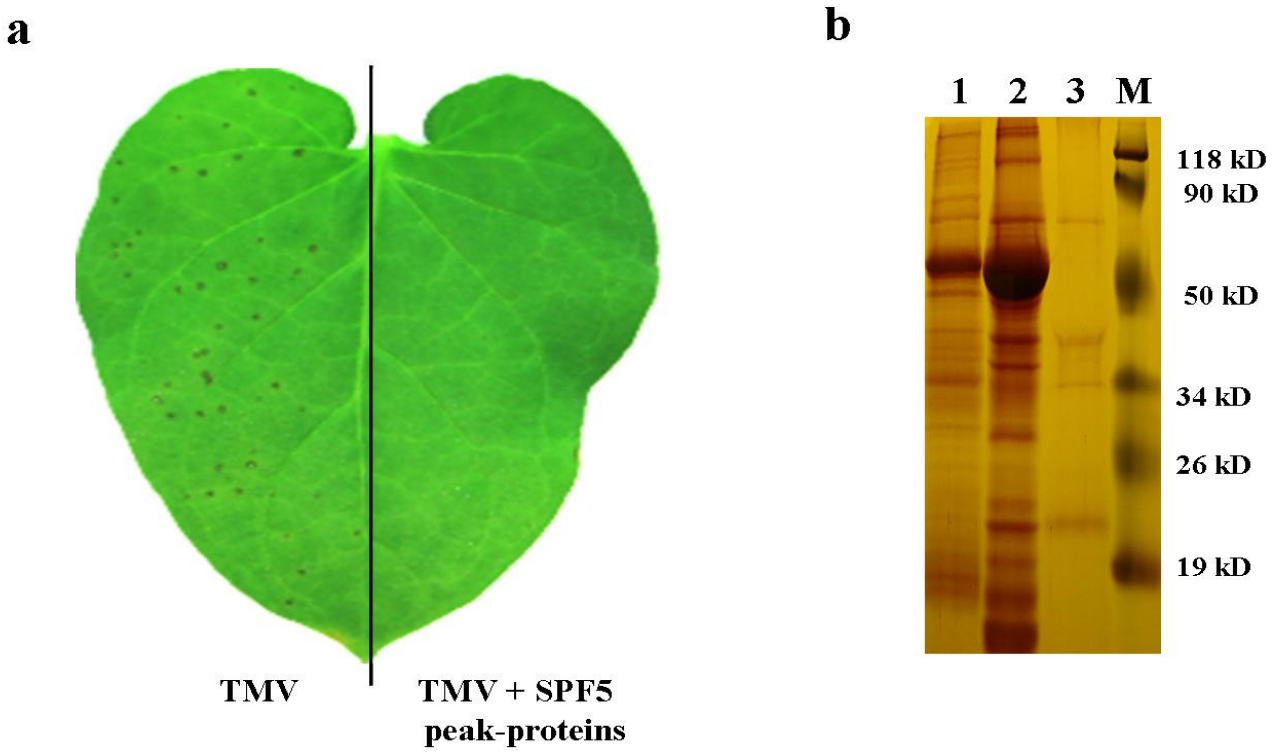

Figure 2. Biochemical properties of the SPF5 peak-proteins. (a) The inhibitory effect of SPF5 peak-proteins against TMV on the leaves of *N. glutinosa*. (b) SDS-PAGE analysis of antiviral proteins from spinach at different stages of purification. 1, Crude extract; 2, 0 to 40% $(NH_4)_2SO_4$ saturated fraction; 3, SPF5 peak-proteins; M, molecular weight marker. The displayed pictures are representative of three independent experiments. TMV, *Tobacco mosaic virus*; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

mixed with TMV and incubated for 30 min. The mixtures were mechanically inoculated onto adaxial half-leaves of *N. glutinosa* as treatment, while the other half part of the leaves were inoculated with TMV solution diluted with the same volume of phosphate buffer buffer as the negative control. The inhibitory rate was only 24.72% when 3.125 μg/mL SPF5 peak-proteins were used, and the antiviral activity was up to 94.35% with 50 μg/mL SPF5 proteins. The results demonstrate that the inhibitory activities of SPF5 peak-proteins against TMV are positively relative to their concentrations (Table 3). The medium inhibitory concentration (IC_{50}) of SPF5 fraction was 10 μg/mL.

SPF5 peak-proteins function against TMV at the infection sites

The half leaves of *N. glutinosa* were rubbed with 50 μg/mL SPF5 peak-proteins without carborumdum and the other half leaves were rubbed with phosphate buffer as

Table 3. The inhibitory effect of SPF5 peak-proteins of different concentrations against TMV *in vitro*.

Sample concentration (μg/mL)	Average lesion number[a]		Inhibitory rate (%)
	Treated	Control	
50	2.67	47.17	94.35
25	5.33	37.00	85.59
12.5	15.00	44.25	66.10
6.25	29.75	52.50	43.33
3.125	33.50	44.50	24.72

[a]Mean number of lesions on 9th to 10th leaves of nine tested plants from three independent experiments. TMV, *Tobacco mosaic virus*.

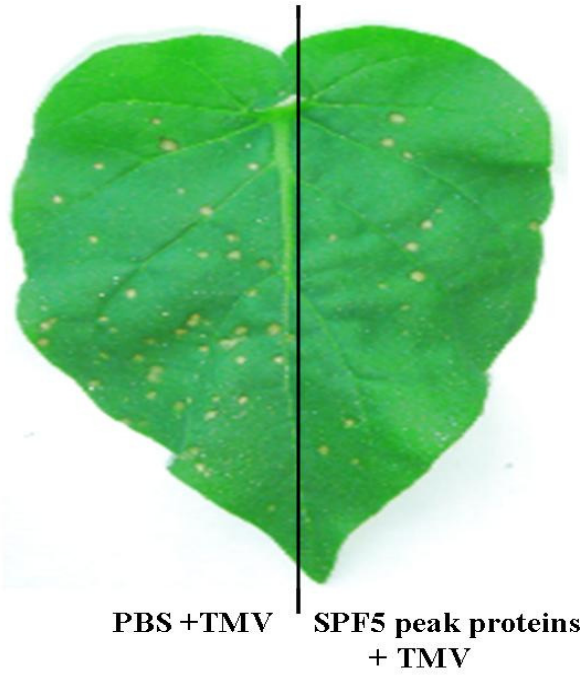

Figure 3. The inhibitory effect of SPF5 peak-proteins against TMV at the infection sites. TMV was inoculated on the right side of the leaves of *N. glutinosa* after pretreatment by SFP5 peak-proteins for 9 h. The left side was control with pretreatment by PBS buffer. The displayed pictures are representative of three independent experiments. TMV, Tobacco mosaic virus; PBS, phosphate buffered saline.

control. Leaves after pretreatment were inoculated with TMV at different time intervals. At 2, 5 and 9 h after the pretreatment, the inhibitory rates of TMV were 93.86, 83.33 and 81.82%, which demonstrated that the SPF5 peak-proteins could prevent TMV infection when it was sprayed on plant in advance (Figure 3 and Table 4).

Inhibitory activity against TMV replication by SPF5 peak-proteins

Growing leaves of *N. glutinosa* were inoculated both leaves with equal volumes of TMV. The right adaxial sides of the leaves were treated with the SPF5 peak-proteins at different time intervals. Low inhibitory activity (46.86%) was detected when 50 μg/mL SPF5 peak-proteins were used 2 h after TMV inoculation. Moreover, the inhibitory activities of SPF5 peak-proteins against TMV replication become fainter as the time interval increased (Table 5). After 9 h, the inhibitory rate was only 26.89% (Figure 4). The results indicate that SPF5 peak-proteins had little inhibitory activity against TMV replication.

DISCUSSION

Plants themselves produce a lot of actively physiological components, including organic chemicals and proteins. About one third pharmaceuticals and half of the total drug industry output contain extracts from plants. Exploitation of eco-friendly antiviral plant extract is a main focus when we seek ways to control plant virus diseases. Many of the antiviral components are plant proteins, such as PAP (Irvin et al., 1980; Chen et al., 1991) and mirabilis anti-viral protein (MAP) (Kubo et al., 1990), both of which have high anti-virus activity. A basic protein isolated from the leaves of *C. inerme* could protect plants against CMV, PVY and TMV, and the inhibitory rate was more than 80% (Shelly et al., 2001). Ribosome inactivating protein (RIP) CIP-31 could destroy the coat protein synthesis process at sub-nanogram level without negative effect on the plant growth (Li et al., 2007).

Crude extract of spinach has been found having high antiviral activity (Cho et al., 2000). We isolated a group of TMV inhibitive protein components using fraction precipitation by ammonium sulfate followed by cation-exchange chromatography column chromatography. The active protein group with 94.35% inhibitory for TMV was eluted down at 0.5 M NaCl, which was designated as SPF5 peak-proteins. The components of SPF5 peak-proteins were analyzed with SDS-PAGE and four bands were detected. Therefore, there was probably more than one component responsible for the anti-TMV activity of the protein group.

Table 4. Inhibitory rates of TMV at the infection sites by SPF5 peak-proteins.

Time of pretreatment before TMV inoculation (h)	Average lesion number[a]		Inhibitory rate (%)
	Treated	Control	
2	2.83	46.17	93.86
5	7.00	42.00	83.33
9	10.00	55.00	81.82

[a]Mean number of lesions on 9th to 10th leaves of nine tested plants from three independent experiments. TMV, Tobacco mosaic virus.

Table 5. Inhibitory rates of TMV replication by the SPF5 peak-proteins.

Time after TMV inoculation (h)	Average lesion number[a]		Inhibitory rate (%)
	Treated	Control	
2	23.25	43.75	46.86
5	28.40	45.40	37.44
9	31.00	42.40	26.89

[a]Mean number of lesions on 9th to 10th leaves of nine tested plants from three independent experiments. TMV, *Tobacco mosaic virus*.

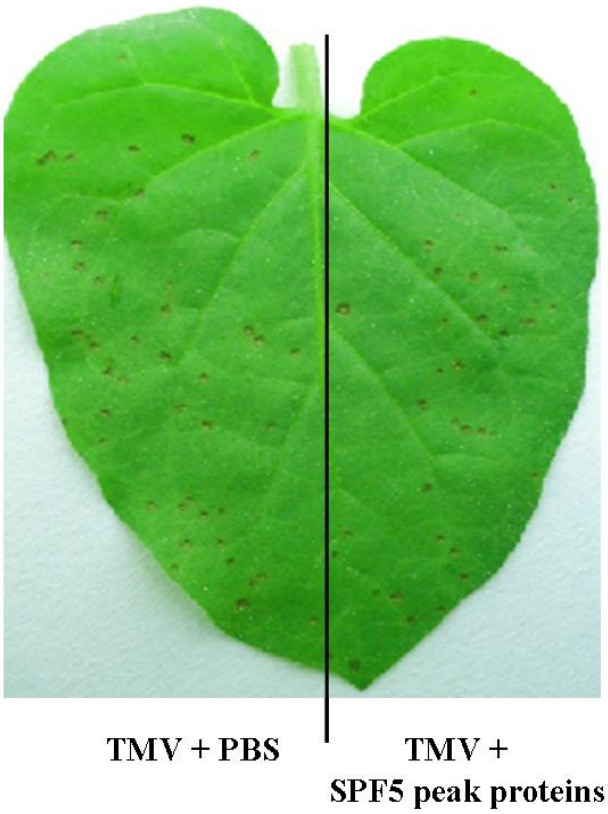

Figure 4. The inhibitory effect of SPF5 peak-proteins against TMV replication. TMV was inoculated on both sides of the leaves of *N. glutinosa*. After 9 h, SFP5 peak-proteins were used to treat the left side leaves and the right side leaves were treated with PBS buffer as controls. The displayed pictures are representative of three independent experiments. TMV, *Tobacco mosaic virus*; PBS, phosphate buffered saline.

The antiviral components from plant could interact with virus directly (Chen et al., 2006), or they could occupy the virus infection sites (Baranwal et al., 1992). Otherwise, they might inhibit the replication of virus, either by interfering formation of the virus coat (Jiang et al., 1996; Shen et al., 2005) or by inhibiting the synthesis of virus protein and RNA (Hou et al., 2000). Our results *indicate* that SPF5 peak-proteins could inhibit the virus activity in a concentration-dependent way. TMV infection progress was slowed down when tobacco leaves was pretreated with SPF5 peak-proteins in advance. However, SPF5 did not have high inhibitory activity against TMV replication and proliferation when TMV had already infected tobacco plants. Therefore, SPF5 peak-proteins could not only destroy the interaction between TMV and its infection sites *in vitro*, but also might inhibit TMV replication *in vivo*. The former hypothesis should be the main reason for the anti-TMV ability of SPF5 peak-proteins.

Conclusion

In our study, SPF5 peak-proteins with TMV inhibitory activity was isolated from spinach. The *in vitro* tests showed that SPF5 peak-proteins had strong activities against TMV infection, but its inhibitory activity on TMV replication was not apparent. The functional protein in the SFP5 peak-proteins should be isolated further, and the molecular mechanisms under the phenomena would be the focus in the future research. Overall, identification of a small group of proteins with anti-TMV activity in this study provides more information for resistant proteins in plants and will advance our understanding of the application of resistant proteins for biological defense.

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