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Characteristics of Tobacco Mosaic Virus Isolated from Wasabi (*Eutrema wasabi*) in Korea

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Wasabies showing mosaic symptoms were collected and extracted for virus purification. Tobacco mosaic virus (TMV) was identified as casual agent by electron microscopy and nucleic acid and coat protein analyses. TMV strains were determined by enzyme-linked immunosorbent assay (ELISA). TMV was identified as W and C strain in wasabi. The results of host reaction indicated that this virus induced local lesions on *Nicotiana tabacum* cv. Bright Yellow and *N. glutinosa*, leaf spots on *Chenopodium amaranticolor* and mosaic symptoms on wasabi. Rod shape virus particles were observed and was about 300 nm in length. About 6.5 kb single RNA molecule was observed from extracted viral RNA sample and 26 KDa coat protein was detected in denatured acrylamide gel. Infection ratio of TMV was 8% for the first cultivation year, but was 22% for the second year when TMV-W antiserum was used. The results of this experiment showed that infection ratios of both TMV-W and TMV-C strains were higher compared to that of TMV-P strain.

Keywords **:** coat protein, TMV strains, wasabi plants.

Wasabi (*Eutrema wasabi* Maxim.) was known as a perennial herbaceous plant which is belong to cruciferae family. It is generally used for manufacture materials including its leaves and roots. However, the rhizomes and flower stalks of wasabi have mainly been used for aromatic essence. It is mainly cultivated in Japan, Taiwan and Korea. The propagation of wasabi can be divided into nutrition and seedling propagation. The nutrition propagation has demerits of low proliferation rate and high possibilities of various diseases, such as soft rot, black leg and virus disease. Chonbuk-Muju, a cultivation area of wasabi in Korea, have also used a nutrition or seedling propagation as a proliferation method. The wasabi, consecutively cultivated using nutrition propagation bulb, was apparently seemed to be good, but showed degenerative phenomenon caused by decreased growth rate and virus infection. Because of these cultural characteristics, particularly in case of dividing, virus infections are becoming influential as one of the biggest problems. Viruses reported from diseased wasabi showing curl symptom include tobacco mosaic virus (TMV; Tochihara et al., 1964), cucumber mosaic virus (CMV; Komuro and Tochihara, 1966), turnip mosaic virus (TuMV; Tochihara et al., 1964), broad bean wilt virus (BBWV; Maoka et al., 1990) and wasabi latent virus (WLV; Kishira et al., 1992) in Japan, and alfalfa mosaic virus (AMV; Fletcher, 1989) in New Zealand.

TMV had a wide host range including crucifer flower, bottle gourd and eggplant families, and damaged seriously to many plants (Oshima et al., 1974). Many TMV strains have been reported and showed different pathogenesis. Thus, the control of TMV had a lot of difficulties in the field. TMV-infection of wasabi caused growth inhibition and subsequently decreased it's production (Komuro and Tochihara, 1996: Tochihara et al, 1964). However, there are only a few reports on wasabi virus diseases in Korea. Furthermore, there is neither a report on TMV nor a study on the TMV-strains on wasabi. It is required to make a precise examination of classification and identification of wasabi virus-germs and strains to establish good control stratigies. Therefore, this study was conducted to identity TMV infection condition and strains of virus obtained from cultivated wasabi in Chonbuk-Muju area.

Materials and Methods

Virus-isolation and plant testing. Virus isolation was performed by selecting wasabi showing obvious symptoms and used as a test materials. Those were cultivated on Chonbuk-Muju from 1995 to 1996, Sap inoculation was performed by adding 0.1 M phosphate buffer (pH 6.8; 20X v/w) to 0.5 g of an infected samples showing mosaic yellowing and curl symptoms. Extracted wasabi samples were inoculated into healthy plants by mechanical inoculation. *Nicotiana tabacum* cv. Bright Yellow, *N. glutinosa* and *Chenopodium amaranticolor* were used as indicator plants. Symptom developments on those plants were observed for 2-3 weeks after inoculation.

Observation of an electron microscope. Dip method was used to measure the size of a virus. Briefly, diseased leaves were cut down to 3 mm and a drop of 2% phospho-tungstic acid was added. Ground samples were soaked in a 200-mesh grid covered with formvar film for 3 seconds, absorbed PTA-liquid remaining

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on grid with filter paper and dried for 1 min. Then virus particles were observed using EM (Carl Zeis EM 10C, Germany).

ELISA test. Twenty different infected samples were used for ELISA test. The antisera used for this test were obtained from Japan National Agricultural Research Center (TMV-W), Koto of Hokkaido Agricultural Research Center (TMV-C), and Japanese Plant Quarantine Office (TMV-P). The titer for these antisera (TMV-W, TMV-C and TMV-P) was 640X, and the conjugate titer was 800 times. ELISA was performed in accordance with the methods of Clark and Adams (1977). Reaction was measured using microplate reader (BioRed model 450) at the light absorption degree of 405 nm.

Purification. Purification of TMV-W was performed using the methods of Takahashi and Ahohara (1990), with minor modification. TMV infected wasabi (cv. Dalma) leaves, propagated by sap-inoculation, were designated as a testing material. Purification was performed by putting 0.1 M sodium citrate buffer (pH 6.8, 3X w/v) in the infected leaves, grinding them for 30 min in a 4°C cold room, and then filtrating through cheese cloth. After stirring up 1/5 carbon tetrachloride and 1/5 ethyl-ether in filtrate for 10 min, several centrifugation steps were followed. The final deposit in CsCl (concentration 1.28 g/cm^3) was obtained by density gradient centrifuge for 16 hr at 120,000 g. A virus band was selected and it was refloated in 0.1 M citric acid buffer.

Extraction of nucleic acid. Virus particles (2 mg/ml) were treated 0.5% SDS and proteinase K (1 mg/ml) for 20 min at 37°C. Thereafter, these reactants were extracted with TE saturated phenol (pH 7.0) and chloroform twice each, and virus RNA was precipitated with ethanol. Amount of extracted RNAs was measured by UV-spectroscopy.

Electrophoresis of coat protein and nucleic acid. SDS-PAGE electrophoresis was performed for virus coat protein (Laemmli, 1970). For coat protein analysis, electrophoresis was performed for 6 hr at 18 mA using 12.5% polyacrylamide gel. And the gel was stained with 0.2% coomassie brilliant blue R250 dye-solution for 1 hr. For electrophoresis of nucleic acid of a virus, purified virus particles were treated with 1 μl of SDS (10%) and isolated nucleic acid were separated on 1.2% agarose gel.

Investigation of the rate of infection on the field. To investigate an annual rate of TMV-W infection, the 5 different fields in Muju, Chonbuk were investigated for the 1st- and 2nd-year cultivation periods.

Results and Discussion

Symptoms. The infected leaves by TMV were generally curled in comparison with normal leaves. They showed mosaic symptoms and became thicker with narrow intervein spaces (Fig. 1A). The newly grown leaves were characterized by the formation of irregular surface and green-faded spots. Infected leaves also formed irregular surface with poor growth and showed a curl phenomenon as previously described (Komuro and Tochihara, 1966). Chlorotic spots were also observed in infected leaves (Tochihara et al., 1964). In general, the symptoms observed in this study were similar to many reported TMV symptoms.

Symptoms on on indicator plants. Purified virus was inoculated into wasabi, *Nicotiana tabacum* cv. Bright Yellow, *N. glutinosa* and *Chenopodium amaranticolor.* As shown in Table 1, virus infection caused local legions in *N. tabacum* cv. Bright Yellow and *N. glutinosa*, small spots in *C. amaranticolor*, and mosaic symptoms in wasabi. These results were in agreement with those of previously reported (Komuro and Tochihara, 1966; Oshima et al. 1974; Tochihara et al., 1964).

Observation by electron microscopy. Extracted virus particles from infected wasabi leaves were observed by an electron microscope using a dip method. Typical rod shape virus particles were found and was a 300 nm long in size (Fig. 1B). Size and shapes for TuMV and TMV particles isolated infected wasabi were identified into 750 nm filament and 300 nm rod shape, respectively. The observed

Table 1. Reactions of indicator plants induced by TMV isolated from wasabi

Indicator plant	Reaction[a]
Nicotiana glutinosa	L
N. tabacum cv. Bright Yellow	L
Chenopodium amaranticolor	S
Eutrema wasabi	M

[a]L; local lesion, S; small local lesion, M; mosaic symptom

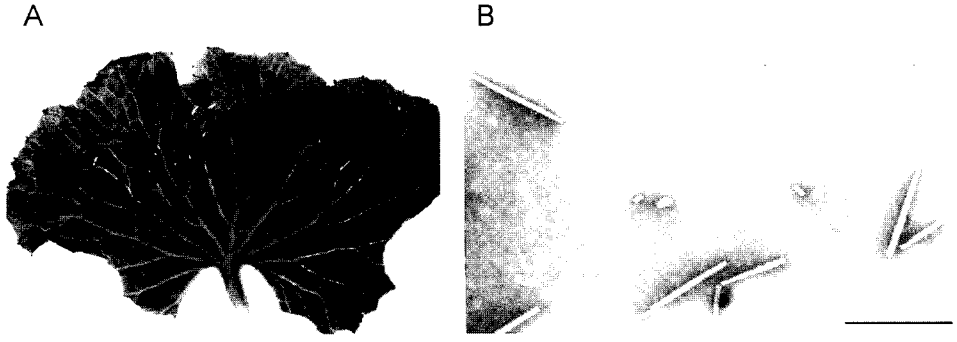

Fig. 1. A: Leaf of wasabi infected with TMV, B: Particles of TMV by dipping method from infected wasabi. The scale bar represents 0.5 μm.

Table 2. Reactions by ELISA of virus infected samples with TMV strains at Muju areas in Korea, 1995-1996

Sample No.	Antibody[a]		
	anti TMV-W	anti TMV-C	anti TMV-P
1	1.147[b]	0.340	0.022
2	1.124	0.210	0.013
3	1.217	0.209	0.006
4	0.467	0.911	0.016
5	1.201	0.201	0.005
6	1.224	0.307	0.005
7	0.140	0.101	0.021
8	0.248	0.254	0.052
9	1.080	0.248	0.233
10	0.960	0.215	0.008
11	0.987	0.305	0.000
12	1.040	0.306	0.045
13	1.134	0.212	0.002
14	0.963	0.411	0.063
15	1.273	0.412	0.010
16	0.820	0.717	0.019
17	1.110	0.612	0.009
18	0.770	0.313	0.020
19	1.072	0.347	0.013
20	1.174	0.414	0.020
21	0.007	0.004	0.022

[a]TMV-W; anti tobacco mosaic virus from wasabi, TMV-C; anti tobacco mosaic virus from crucifer, TMV-P; anti tobacco mosaic virus from pepper, No.21: healthy plant.
[b] The number is ELISA-reader based.

virus particles were very similar to those of TMV-W.

Serological detection of TMV strains. Results obtained from 20 different infected samples using 3 different TMV antisera were described in Table 2. There were different serum reaction between TMV-strains, whereas TMV-W, TMV-C and TMV-P responded positive reactions for all antiserums. In reaction of antiserums, Nineteen TMV-W and TMV-C, showed a positive reaction of twenty tested samples. In contrast, only one TMV-P showed a positive reaction of all tested samples. Most TMV-W and -C were infected in a mixed condition, but only one TMV-P was infected into mixed types of virus. In this experiment, TMV-W showed a stronger reaction than TMV-C or TMV-P and contained the most serious one. In view of serological relations, it appeared that TMV-W formed higher interrelation with TMV-C than that of TMV-P. Kashiwazaki et al. (1990) made a comparative analysis of serological interrelations of five TMV antiserums (TMV-W, TMV-C, TMV-P, TMV-OM, TMV-L) and showed that the antiserums of TMV-W and TMV-C showed strong reactions, while TMV-OM, TMV-L and TMV-P showed weak reactions. The test result obtained from this study indicated similar reactions to the previous reports. Therefore, it could be concluded that the virus-diseases developed from this study were mainly caused by TMV-W strain. When several TMV strains were coinoculated into wasabi (Kawano et al., 1993), TMV-W and TMV-C caused the whole-body infection and simultaneous two-strain virus infection, increasing the possibility of their propagation.

Nucleic acid and coat protein analysis. Viral RNA was extracted from purified virus and loaded onto agarose gel along with RNA extracted from TMV-Japanese strain. Both of them showed a similar size RNA band of about 6.5 kb (Fig. 2-A). Coomasie stained coat protein bands separated on polyacrylamide gel are shown in Fig. 2-B. Both virus obtained from Muju, Chonbuk and TMV-Japanese strain showed similar size single band of about 26 kDa. These results suggested that the virus isolated from wasabi

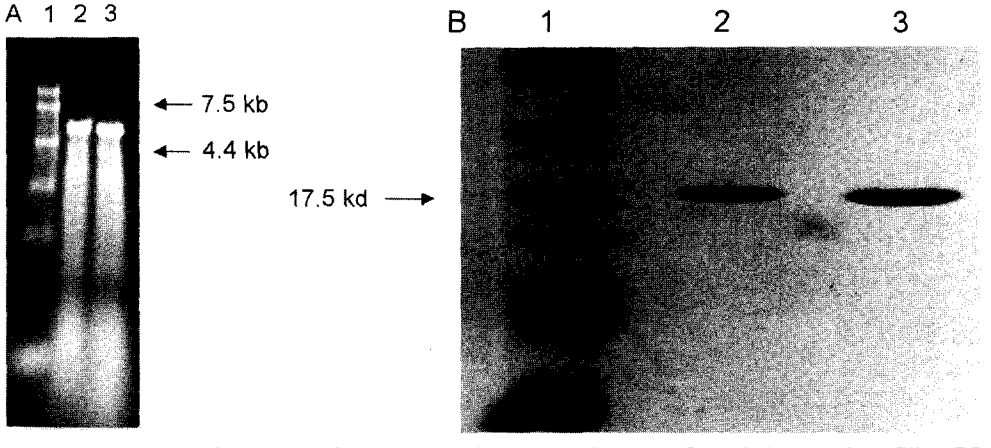

Fig. 2. A: Electrophoretic patterns of viral RNAs of TMV on 1.2% agarose gel. Lane 1. RNA ladder marker (Gibco BRL), Lane 2: TMV (Korean Muju isolate), Lane 3: TMV (Japanes Tochiki isolate). B: Electrophoretic patterns of the TMV coat proteins. Lane 1: rainbow maker protein, Lane 2: TMV (Korean Muju isolate), Lane 3: TMV (Japanese Tochiki isolate).

Table 3. Infect ratio of cultivation periods against tree TMV strains antisera in the ELISA test

Antibody	Cultivation period	
	1st year	2nd year
TMV-W	4/50 (8%)	11/50(22%)
TMV-C	2/50 (4%)	10/50(20%)
TMV-P	1/50 (2%)	2/50(4%)

cultivated in Korea may be TMV. Whether there is a mixed infection as suggested in some of samples need to be determined.

Annual infection ratio. Effects of consecutive cultivation in the field on virus infection ratio were determined using ELISA. Results were summarized in Table 3. After showing seed to field, fifty samples were investigated in autumn, 1995 and 1996. The virus infection was significantly increased in the second year. For example, infection ratio for the first year was 8% and increased to 22% in the second year when TMV-W antiserum was used. Therefore special attention and filed maintenance needed for the steady production of wasabi. Komuro and Tochihara (1966) reported that TMV infection ratio was upto 32% in certain areas and the result of this study also support for that reports.

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