Epidemiological Aspects of the Japanese Tobamovirus Strain, *Pepper Mild Mottle Virus* (PMMoV) Infecting the L^2 resistance Genotype of Green Pepper (*Capsicum annuum* L.)

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To understand the epidemiological aspects of tobamovirus infecting the *L* resistance genotypes of green pepper, fifteen isolates were collected from geographically different fields and were characterized by their biological and biochemical properties. All isolates infected L^1 and L^2 plants systemically, but were localized in L^3 and L^4 plants. The symptomatology on several test plants and the reactivity to an antiserum showed that they were identical to that of a Japanese strain of *pepper mild mottle virus* (PMMoV–J). The viral infection was also confirmed by a reverse transcription and polymerase chain reaction (RT–PCR) with oligonucleotide primers that amplify the coat protein gene of PMMoV–RNA. On the other hand, the RT–PCR allowed us to detect PMMoV in seeds of some commercial cultivars of green pepper. Viruses isolated from the seeds could infect L^2 plants systemically. Further analysis of the nucleotide sequence of the predicted coat protein gene revealed that the isolates from the commercial seeds were identical to that of PMMoV–J. These results indicated that the L^2 resistance-breaking tobamovirus has prevailed in fields of green pepper in Japan, and that infected seeds may be one of the initial sources of the viral infection.

Key words : *Capsicum annuum* L., *Pepper mild mottle virus* (PMMoV), RT–PCR, resistance-breaking tobamovirus

Introduction

Capsicum spp. plants have resistance against tobamoviruses such as *Tobacco mosaic virus* (TMV) and *Tomato mosaic virus* (ToMV), which are manifested by the appearance of necrotic local lesions at the infection site[3,9]. The resistance is conferred by four different genes at *L*-locus known as L^1, L^2, L^3 and L^4, respectively[3,9]. Over the past decade, however, new tobamoviruses or strains infecting the *L*-resistance genotypes have been described in different parts of the world; in Australia[17], France[7], Italy[1,23], The Netherlands[18] and Spain[6]. These resistance-breaking tobamoviruses have been characterized by their biological properties, and most viruses have been classified into four subgroups of pathotype P_0, P_1, $P_{1,2}$ and $P_{1,2,3}$, based on their ability to overcome resistance governed by L^1, L^2 and L^3, respectively[1,2,5–7,23]. At present, the virus of P_1 pathotype is referred to as

Paprika mild mottle virus (PaMMoV)[5], and the viruses of $P_{1,2}$ and $P_{1,2,3}$ pathotype are termed as *Pepper mild mottle virus* strain S (PMMoV–S)[1,6] and strain I (PMMoV–I)[2], respectively, since they were first described in Spain and Italy, respectively. Such a classi-

fication now become available[1,2,5-7,23], because these tobamoviruses are hard to distinguish from each other serologically and morphologically.

In Japan, Nagai *et al.*[15] first reported the occurrence of a new tobamovirus that was able to infect resistant cultivars of green pepper. The virus was serologically distinguishable from both TMV and ToMV even though it shared the same antigenic sites[15]. Since then, the term *Tobacco mosaic virus* pepper strain (TMV-P)[15] has been used for the new virus in Japan. Recently, an isolate of TMV-P has been shown to be similar to a Spanish strain of PMMoV (PMMoV-S), based on the analysis of the whole nucleotide sequence[8,10,16], and the virus is named as a Japanese strain of PMMoV (PMMoV-J)[10]. However, there were few descriptive reports covering the epidemiological aspects, including the local or long-range dispersal of the virus. Since this tobamovirus, unlike other economically important viruses such as *Cucumber mosaic virus* (CMV) and *Tomato spotted wilt virus* (TSWV), is not dispersed by insect vectors[9,13], diagnosis and knowledge of the viral distribution would thus be essential to reduce the potential threats in protected crops of green pepper. In this study, we isolated and characterized tobamovirus infecting the *L* resistance genotypes of green pepper, and also described some of the epidemiological aspects of this virus.

Materials and Methods

Virus sources, isolation and propagation

Tobamovirus isolates were collected from infected green peppers grown under plastic houses in Japan, during 1996 and 1997 (Fig. 1). The viruses were isolated by a repeated single lesion isolation on leaves of *Nicotiana glutinosa* L. and were propagated in *N. benthamiana* Domin. as described previously[7,10,15]. Isolates from Chiba, Ibaraki and Miyazaki Prefecture were kindly provided by Dr. T. Takeuchi (Chiba Prefectural Agricultural Experiment Station, Daizenno, Chiba, Japan). An isolate of TMV-P, originally isolated in Chiba Prefecture[15], was used as a reference strain.

Plant materials

Seeds of *Capsicum annuum* L. cv. Shosuke (carrying no resistance gene; $L-/L-$), *C. annuum* L. cv. Verbeterde Glas (L^1/L^1), *C. flutescens* L. cv. Tabasco (L^2/L^2), *C. chinence* L. accession PI152225 (L^3/L^3) and *C. chacoence* L. accession PI260429 and SA815 (L^4/L^4) were sown in 10 cm diameter pots containing sterilized composts and grown in a glasshouse under a natural light. Five to 6 week-old seedlings with 4 to 5 expand-
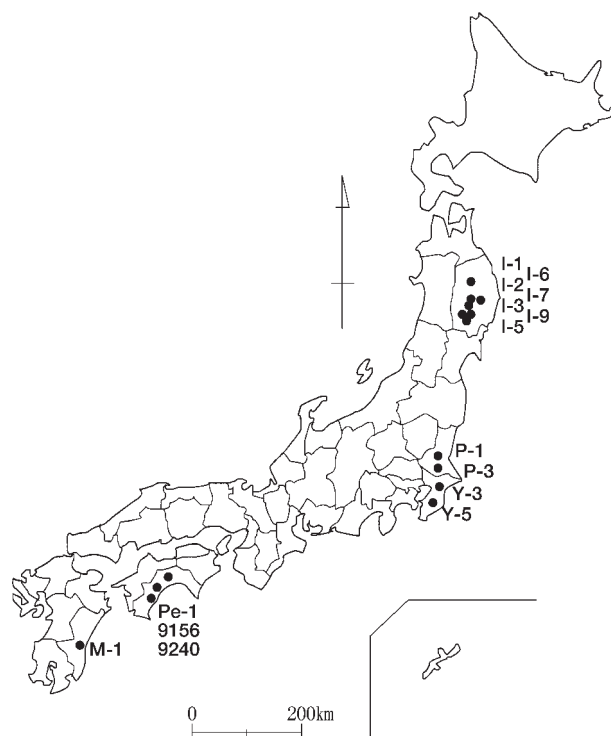
Fig. 1   Geographic origin of tobamovirus isolates used in this study.
Fifteen isolates were collected from Iwate, Ibaraki, Chiba, Kochi and Miyazaki Prefecture. Each isolate was given a reference name in the figure.

ed leaves were inoculated mechanically using 600-mesh carborundum. The inoculated plants were kept in the glasshouse and symptoms were observed for up to 4 weeks.

RNA extraction and RT-PCR

Samples of infected tissues (approximately 100 mg) were ground in liquid N_2 and total RNA was extracted according to the method of Verwoerd *et al.*[22]. Four oligonucleotide primers (20-mer) that amplify the coat protein gene of PMMoV-RNA were designed from sequence information from EMBL, GenBank and DDBJ databases[1,8,10,16] (Fig. 2). First-strand cDNA was synthesized from total RNAs of infected tissues with an avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa) using either 5'-GAG TTA TCG TAC TCG CCA CG-3' (designated as R1), 5'-TCC CGT GCC ACG AAC TAA CT-3' (R2) or 5'-CGA GAA GTG CCG ACA CTA GA-3' (R3), complementary to nucleotide position at 6198 to 6217, 6079 to 6098 or 5921 to 5940 of PMMoV-RNA. The synthesized cDNAs were further mixed for specific amplification with an oligonucleotide, 5'-AGA ACT CGG AGT CAT CGG AC-3' (F1), corresponding to the nucleotide position at 5642 to 5661, in the reaction mixture (100 μl)

Fig. 2   TEM observation of tobamovirus isolate and its infectivity against *Capsicum* varieties carrying *L* resistance gene.
  A   TEM observation of tobamovirus isolate. Bar＝100 nm.
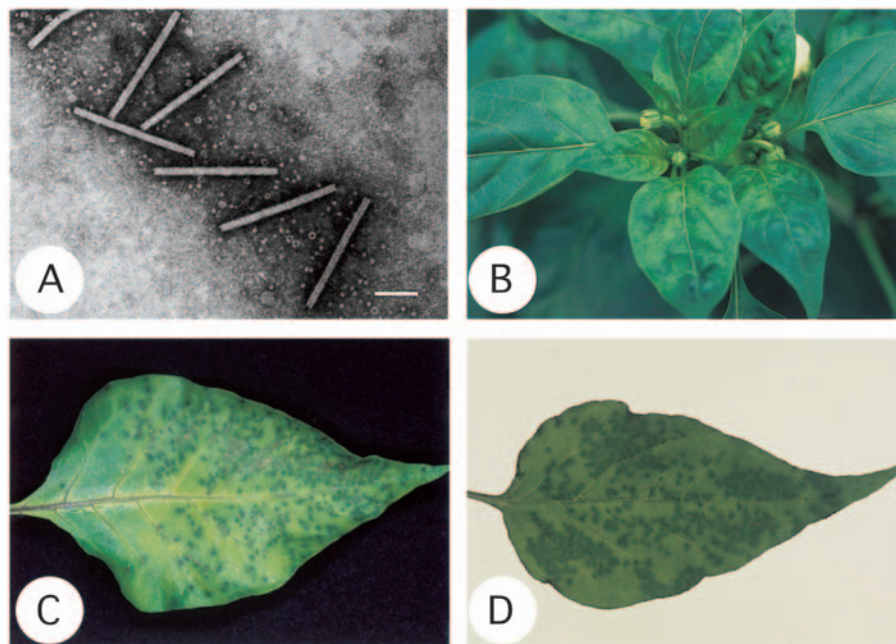 B   Symptoms on green pepper (carrying L^2 gene) infected by a PMMoV isolate, I-6. The symptom was observed 3 weeks after inoculation.
  C   Necrotic local lesions developed on inoculated leaves of *C. flutescens* L. PI152225 (L^3/L^3).
  D   Chlorotic spots developed on inoculated leaves of *C. chacoence* L. PI260429 (L^4/L^4).

Table 1   Symptomatology of tobamovirus isolates used in the study

Plants	Cultivars/accessions	Symptoms[a]
Chenopodium amaranticolor		CS/−
C. quinoa		NS/−
Cucumis melo L.	Prince PF	−/−
Cucumis sativus L.	Hokushin	−/−
Phaseolus vulgaris L.	Kintoki	−/−
Lycopersicon esculentum Mill.	Momotaro	−/−
Nicotiana benthamiana Domin.		−/M
N. glutinosa L.		NS/−
N. tabacum L.	Bright Yellow	NS/−
Vigna unguiculata Endl.	Kurodane-sanjaku	−/−
Capsicum annuum L.	Shosuke and 7 cultivars[b]	−/M
C. annuum L.	Verbeterde Glas[c]	−/M
C. flutescens L.	Tabasco[d]	−/M
C. chinence L.	PI152225[e]	NS/−
C. chacoence L.	PI260429[f] and SA815[f]	CS/−

[a]Local reaction/systemic reaction. M; mosaic, CS; chlorotic spot, NS; necrotic spot, −; symptomless
[b]Kyoyutaka, Hohryoku, Ryokuho, Sakigake, Shimohusa No. 2, Shishito and Suigyoku No. 2
[c–f]Resistant genotype conferred by either L^1, L^2, L^3 or L^4 gene, respectively.

containing 10 mM Tris/HCl (pH 8.9), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM of dNTP and 2.5 U *Taq* DNA polymerase (TaKaRa). For detection of the minus strand RNA during the replication process, cDNA was first synthesized with the homologous F1 primer and was then amplified in the presence of R1 primer. The PCR was done for 25 cycles with the program (94 ℃, 1 min; 45 ℃, 1 min; 72 ℃, 1 min) with a TaKaRa

TP3000 thermal cycler. Viral RNAs of other *Capsicum*-infecting strains, TMV-OM and TMV-U[14] were also used as template to examine the specific amplification. Ten μl of each reaction mixture was fractionated on 1% agarose gel and the gel was stained with ethidium bromide.

DNA sequencing

Amplified double-stranded DNAs were sequenced directly by the dideoxy chain termination method with an ABI PRISM™ Cycle Sequencing Kit (Applied Biosystems Co., Ltd.). The sequences were determined with an Applied Biosystems 373A automated DNA sequencer and the data were assembled and analyzed with the GENETYX™ program (Software Development Co., Ltd.).

A tissue printing immunoassay

A tissue printing immunoassay with an antiserum raised against the purified PMMoV-J (donated by Dr. S. Tsuda, Ibaraki Plant Biotechnology Institute, Nishi-Ibaraki, Ibaraki, Japan) was employed to detect virus infection, according to the method described by Lin *et al.*[11]. Detached leaves from infected plants were rolled, cut across the main vein with a razor blade and the cut surface was pressed onto a polyvinylidene difluoride (PVDF) membrane (Millipore). The blots were blocked with 3% (w/v) gelatin-phosphate buffer (pH 7.4) for 1h at room temperature and incubated for 2h with the antiserum (1:10,000). The blots were reacted with goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) for 30 min and were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).

Isolation of viral RNA from seeds

Twenty seeds were randomly selected; half (10 seeds) were used for an RT-PCR analysis and the rest were for an inoculation test. The seeds for the RT-PCR analysis were ground in liquid N_2 and RNA extracts were prepared as described above. In some expriments, RNA extracts from seeds of rice (*Oryza sativa* L. cv. Nippon-bare) and tomato (*Lycopersicon esculentum* Mill. cv. Momotaro) were used for the negative controls. For the inoculation test, seeds were homogenized with 1ml of 0.1M phosphate buffer (pH 7.4) and the homogenates were used for inoculation with *N. glutinosa*. Viruses were isolated from necrotic local lesions by a repeated single lesion isolation on *N. glutinosa* and were propagated in *N. benthamiana*.

Electron microscopy

Leaf sap of infected leaves was examined with a JEOL JEM-100C electron microscope at 80kV after staining with 2% (w/v) phosphotungstic acid (pH 7.0).

Results

Symptomatology on test plants

All field isolates induced either chlorotic or necrotic local lesions on inoculated leaves of *Chenopodium amaranticolor* or *C. quinoa*, respectively (Table 1). *Cucumis melo* L. cv. Prince PF, *C. sativus* L. cv. Hokushin, *Phaseolus vulgaris* L. cv. Kintoki, *Lycopersicon esculentum* Mill. cv. Momotaro and *Vigna unguiculata* Endl. cv. Kurodane-sanjaku were not infected by any isolates. On *N. glutinosa* L. and *N. tabacum* L. cv. Bright Yellow, all isolates produced distinct necrotic local lesions which were much smaller than those produced by the common strain of TMV (TMV-OM). *N. benthamiana* was systemically infected with all isolates. The symptomatology of their isolates was
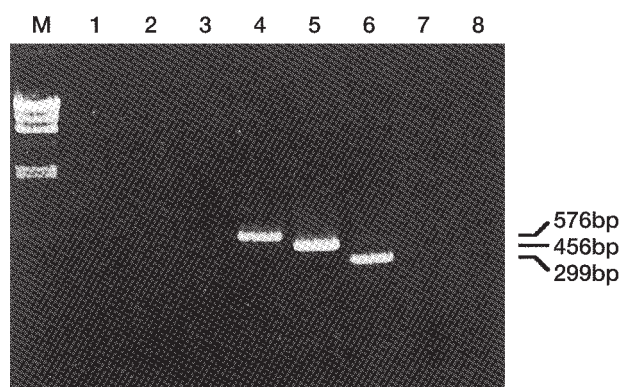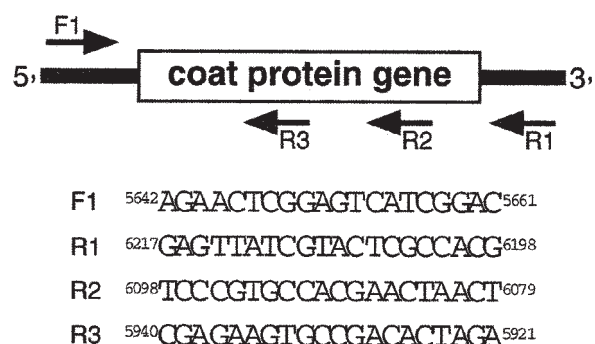
Fig. 3   A PCR-based amplification of PMMoV-RNA.
The RT-PCR was carried out with three primer sets (R1 and F1, R2 and F1, R3 and F1) as described in the figure. Lane M, λDNA digested with *Hind* III. Lanes 1, 2 and 3 were amplifications of RNA extracts from healthy leaves in the presence of R1, R2 or R3, respectively. Lanes 4, 5 and 6 were amplifications of RNA extracts from infected plants. Each primer set produced DNA fragments with the expected sizes of 576, 456 or 299bp, respectively. Lanes 7 and 8 were from the RNAs of the common strain of TMV (TMV-OM) and TMV-U in the presence of R1 primer.

similar to that reported for a Japanese strain of PMMoV (PMMoV–J)[10,15] and other foreign L resistance-breaking tobamoviruses[6,7] (Table 1). Electron microscopic observation showed that rod-shaped particles with approximately 300 nm in length and 18 nm in width were abundantly observed in negatively stained leaf suspensions from *N. benthamiana* (Fig. 2A).

Infection on Capsicum *varieties carrying* L *resistance gene*

To further characterize the field isolates, all isolates were tested for infection to *Capsicum* varieties carrying either L^1, L^2, L^3 or L^4 gene. In *C. chinence* carrying L^3 gene, all isolates induced necrotic local lesions on inoculated leaves within 3 days after inoculation (Table 1, Fig. 2C). *C. chacoence* (carrying L^4 gene) responded similarly to the viruses (Fig. 2D). In contrast, all isolates systemically infected *Capsicum* varieties carrying L-, L^1 or L^2 gene (Table 1) and developed mottle symptoms in 2 to 3 weeks after inoculation (Plate IB). Systemic infection in L-, L^1 and

L^2 plants were also confirmed by a tissue printing immunoassay with PMMoV–J antiserum (Fig. 4a) and an RT–PCR analysis (Fig. 3b). These results indicated that all isolates collected here were classified as a $P_{1,2}$ pathotype of *Capsicum* L resistance-breaking tobamovirus.

Specific amplification by an RT-PCR with primer sets that amplify the coat protein gene of PMMoV and their nucleotide sequence analysis

Total RNA was extracted from systemically infected *N. benthamiana* and the RNA was tested for amplification by RT–PCR with three sets of primers specific to the coat protein gene of PMMoV. As shown in Figure 3, the DNA fragments with the expected size (576, 457 and 299 bp) were amplified by the respective primer sets (R1–F1, R2–F1 and R3–F1), although no DNA fragment was observed with RNA from healthy leaves. Similar amplification was also observed with all fifteen isolates. Since the primer sets did not amplify the cDNA fragment in RNAs of TMV–OM and TMV–U (Fig. 3), the RT–PCR was
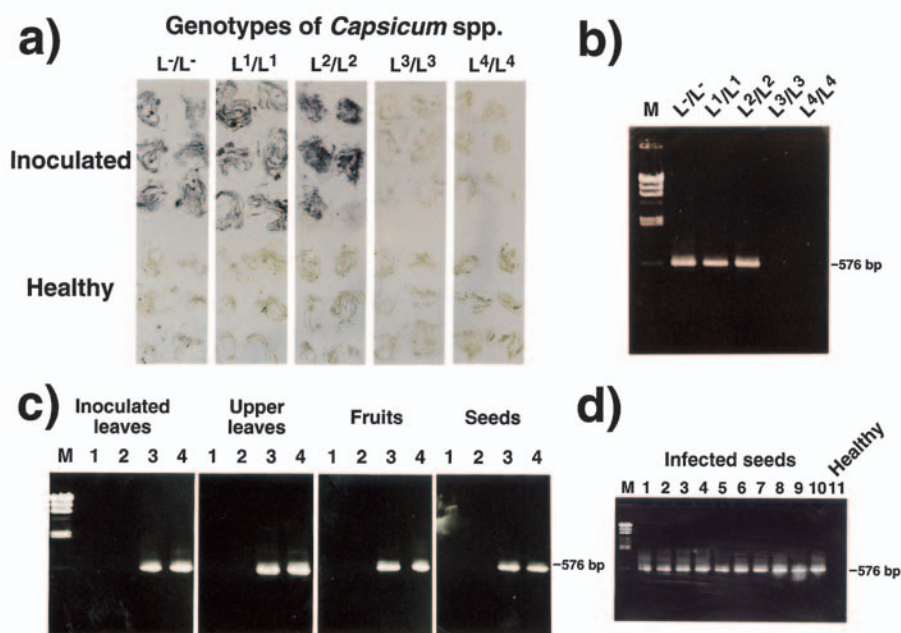
Fig. 4 Systemic infection in L^2–resistance genotype of green pepper.
A field isolate, PMMoV I-6, originally isolated at Iwate Prefecture was inoculated with the *Capsicum* varieties. The virus infection in each *Capsicum* genotype was determined by a tissue printing immunoassay with PMMoV–J antiserum (a) and an RT–PCR assay (b). The upper leaves of each *Capsicum* variety were harvested at 1 and 3 weeks after inoculation, for RT–PCR assay and tissue printing immunoassay, respectively. c), Systemic infection in L^2 plants. Total RNAs were extracted from inoculated leaves, the upper non-inoculated leaves, the growing fruits and the seeds, and were then subjected to the RT–PCR analysis (Lanes 1 and 3). For detection the minus stranded RNAs during the replication process, cDNA was first synthesized with homologous primer (F1) and was subsequently amplified in the presence of R1 primer (Lanes 2 and 4). Lane M, λDNA digested with *Hind* III. Lanes 1 and 2 were amplifications from healthy plants. Lanes 3 and 4 were from inoculated plants. d), Amplification of PMMoV-RNA from single seeds. Ten seeds were tested for the amplification. Lane M, λDNA digested with *Hind* III. Lanes 1-10 were amplifications of RNA extracts from individual seeds. Note that no DNA fragment (576 bp) was observed with healthy seeds (Lane 11).

shown to discriminate PMMoV from other *Capsicum*-infecting tobamoviruses.

On the other hand, fifteen DNA fragments (from 15 field isolates) amplified by the primer set (R1 and F1) were used to determine the nucleotide sequence of the coat protein gene (Fig. 5). The analysis of the predicted coding region of the coat protein gene (474 nucleotides) showed that some isolates (I-3, I-6, P-1, P-3 and Y-3) were identical to that of a Japanese strain of PMMoV (PMMoV-J)[10]. In other isolates, only a few base substitutions were found in the coding region, but these substitutions did not affect the deduced amino acid sequences (Fig. 5). Comparison with other foreign resistance-breaking tobamoviruses showed that all isolates shared the nucleotide sequence identities with PaMMoV[5] (66.3–66.9 %), PMMoV-S[1] (97.5–99.8 %) and PMMoV-I[2] (94.1–94.5 %).

Systemic infection of PMMoV isolate in L^2 plant determined by the RT-PCR

When L^2 plants were inoculated with a PMMoV isolate, I-6, the virus induced mottle symptoms in 2 to 3 weeks after inoculation. The RT-PCR assay allowed detection of the virus infection in growing fruits

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[Field isolates]
                          10        20        30        40        50        60        70        80        90
I-3, I-6, P-1, P-3, Y-3  ATGGCTTACACAGTTTCCAGTGCCAATCAATTAGTGTATTTAGGTTCTGTATGGGCTGATCCATTAGAGTTACAAAATCTGTGTACTTCG
I-5, M-1, 9156           ........................................C.....................A..........
I-1, I-7                 ........................................C.....................A..........
I-2                      ........................................A.....................A..........
I-9                      ........................................C.....................A..........
Pe-1                     ........................................C.....................A..........
9240                     ........................................C.....................T.A........
Y-5                      ......................................................................
PMMoV-J                  ......................................................................
PMMoV-S                  ......................................................................
                          M  A  Y  T  V  S  S  A  N  Q  L  V  Y  L  G  S  V  W  A  D  P  L  E  L  Q  N  L  C  T  S

                          100       110       120       130       140       150       160       170       180
I-3, I-6, P-1, P-3, Y-3  GCGTTAGGCAATCAGTTTCAAACACAACAGGCTAGAACTACGGTTCAACAGCAGTTCTCTGATGTGTGGAAGACTATTCCGACCGCTACA
I-5, M-1, 9156           .......T.............G.....................C..A...........
I-1, I-7                 .......T.............G.....................C..A...........
I-2                      .......T.............G.....................C..A...........
I-9                      .......T.............G.....................C..A...........
Pe-1                     .......T.............G.................A..A..C..A.........
9240                     .......T.............G.....................C..A...........
Y-5                      ..........................................................
PMMoV-J                  ..........................................................
PMMoV-S                  ..........................................................
                          A  L  G  N  Q  F  Q  T  Q  Q  A  R  T  T  V  Q  Q  Q  F  S  D  V  W  K  T  I  P  T  A  T

                          190       200       210       220       230       240       250       260       270
I-3, I-6, P-1, P-3, Y-3  GTTAGATTTCCTGCTACTGGTTTCAAAGTTTTCCGATATAATGCCGTGCTAGATTCTCTAGTGTGTCGGCACTTCTCGGAGCCTTTGATACT
I-5, M-1, 9156           ..............................T...........................
I-1, I-7                 ..............................T...........................
I-2                      ..............................T...........................
I-9                      ..........................................................
Pe-1                     ..............................T...........................
9240                     ..............................T...........................
Y-5                      ..........................................................
PMMoV-J                  ..........................................................
PMMoV-S                  ..........................................................
                          V  R  F  P  A  T  G  F  K  V  F  R  Y  N  A  V  L  D  S  L  V  S  A  L  L  G  A  F  D  T

                          280       290       300       310       320       330       340       350       360
I-3, I-6, P-1, P-3, Y-3  AGGAACAGGATAATAGAAGTTGAAAATCCGCAAAATCCTACAACTGCCGAGACGCTTGATGCGACGAGGCGGGTAGATGATGCGACGGTG
I-5, M-1, 9156           .....T.............A..................
I-1, I-7                 C....T.............A..................
I-2                      C....T.............A..................
I-9                      .....T.............A..................
Pe-1                     .....T.............A.......G..........
9240                     .....T.............A..................
Y-5                      ..........................................
PMMoV-J                  ..........................................
PMMoV-S                  ...........................................C..........
                          R  N  R  I  I  E  V  E  N  P  Q  N  P  T  T  A  E  T  L  D  A  T  R  R  V  D  D  A  T  V

                          370       380       390       400       410       420       430       440       450
I-3, I-6, P-1, P-3, Y-3  GCCATTAGGGCCAGTATAAGTAACCTCATGAATGAGTTAGTTCGTGGCACGGGAATGTACAATCAAGCTCTGTTCGAGAGCGCGAGTGGA
I-5, M-1, 9156           ..........................................................
I-1, I-7                 ..........................................................
I-2                      ..........................................................
I-9                      ..........................................................
Pe-1                     ..........................................................
9240                     ..........................................................
Y-5                      ............................................A.............
PMMoV-J                  ..........................................................
PMMoV-S                  ..........................................................
                          A  I  R  A  S  I  S  N  L  M  N  E  L  V  R  G  T  G  M  Y  N  Q  A  L  F  E  S  A  S  G

                          460       470
I-3, I-6, P-1, P-3, Y-3  CTCACCTGGGCTACAACTCCTTAA
I-5, M-1, 9156           .....................G
I-1, I-7                 .....................G
I-2                      .....................G
I-9                      .....................G
Pe-1                     ......................
9240                     .....................G
Y-5                      ......................
PMMoV-J                  ......................
PMMoV-S                  ......................
                          L  T  W  A  T  T  P  *
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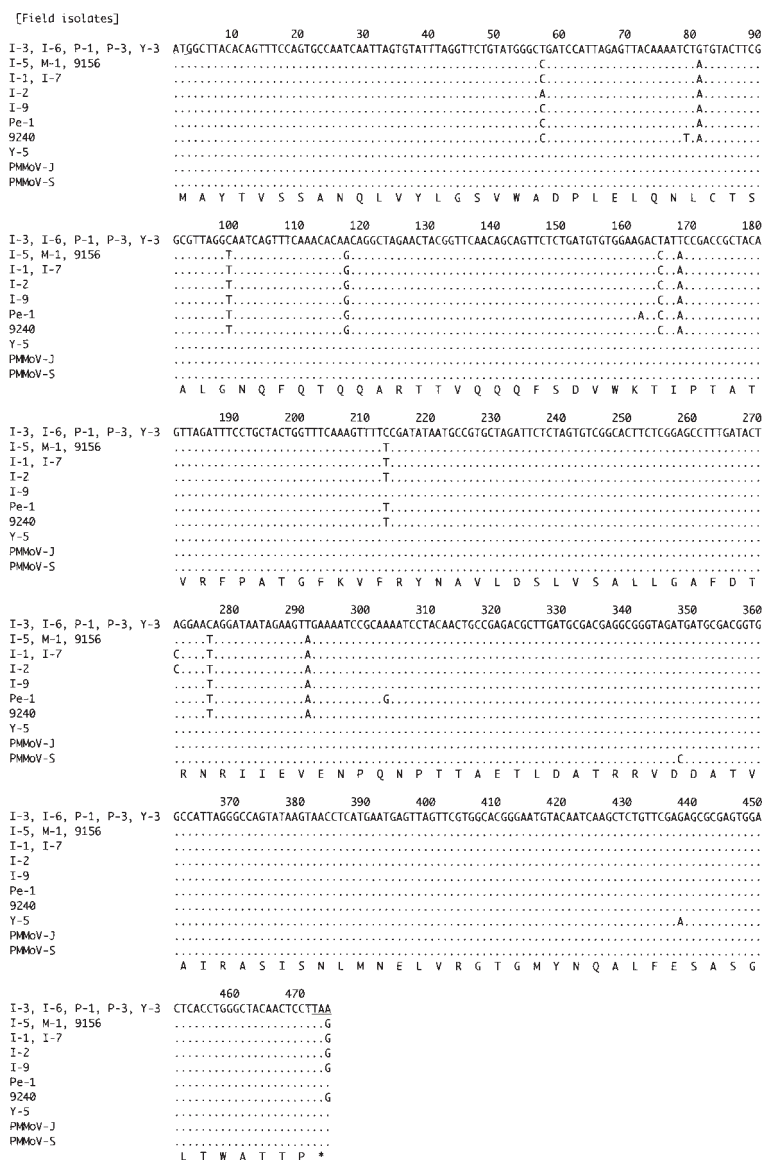
Fig. 5 Nucleotide and the deduced amino acid sequence of the coat protein gene of PMMoV isolates.
The predicted region encoding the coat protein is 474 nucleotides in length and the deduced coat protein consists of 156 amino acids. The initiation codon and the termination codon are underlined. Dotted symbol shows the same base. Any substitutions found in the sequence did not affect the deduced amino acid sequence. The sequences were also compared with those from PMMoV-J[10] and PMMoV-S[1].
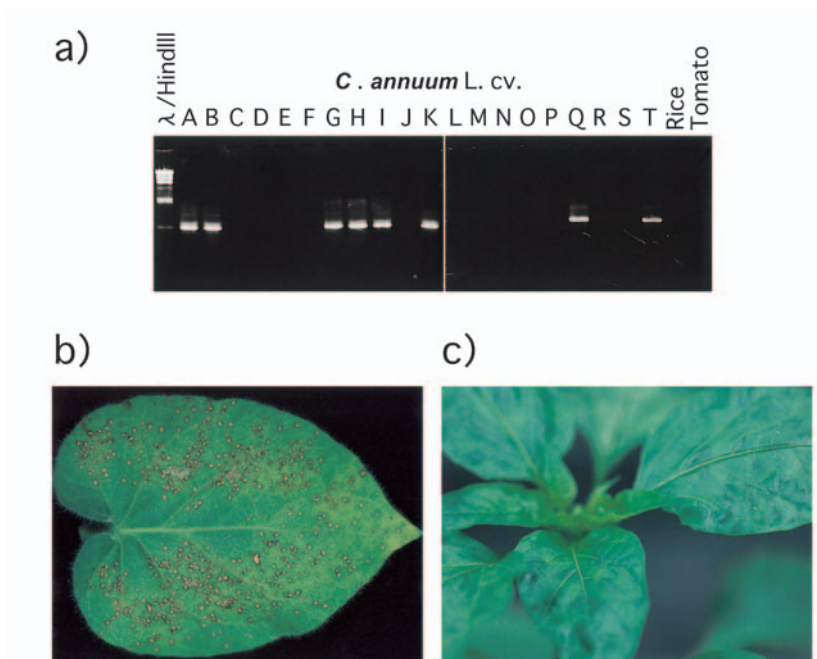
Fig. 6   The presence of infectious PMMoV in commercial seeds of green pepper.
a), Agarose gel electrophoresis of DNAs amplified from RNA extracts of various cultivars. Lanes A - S were amplifications of RNA extracts from various cultivars described in Table 2. RNA extracts from rice and tomato were also used as negative control. b), A typical symptom on *N. glutinosa* induced by the seed homogenates. The symptoms were observed at 3 days after inoculation. c), A mosaic symptom in green pepper (carrying L^2 gene) infected with the virus that had been isolated from seeds. The symptom was observed at 3 weeks after inoculation.

and seeds as well as in the upper leaves (Fig. 4c). The same size of cDNA fragment was also amplified when the first-strand cDNA was synthesized with the homologous primer (F1) having the viral sense sequence (Fig. 4c). This result indicates that the viral RNA replication occurs even in growing fruits and seeds. Figure 4d shows a typical amplification of PMMoV-RNA with seeds harvested from systemically infected plants. This result showed that, in systemically infected plants, the incidence of virus infection in seeds was almost 100 %.

Detection of PMMoV from commercial seeds

Twenty cultivars of commercially available green pepper were tested for amplification of the coat protein gene of PMMoV by using the primer set (R1 and F1). As shown in Figure 6a, DNA fragment specific for PMMoV (576 bp) was observed in 8 of 20 cultivars tested (Table 2, Fig. 6a). When the amplified DNAs from 8 cultivars were sequenced directly, 6 out of 8 sequences were found to be identical to that from PMMoV-J. On the other hand, when tested for the presence of infectious virus by inoculating the seed homogenates to *N. glutinosa* leaves, the homogenates from the six cultivars produced necrotic local lesions on the inoculated leaves (Fig. 6b, Table 2). Virus isolated by a repeated single lesion isolation on leaves

of *N. glutinosa* also gave systemic infection in green peppers (carrying L^2 gene) in 2 to 3 weeks after inoculation (Fig. 6c).

Discussion

Introduction of pepper crops with resistance genes to tobamovirus has been thought to evoke the occurrence of new tobamoviruses or strains that can overcome the resistance[1,5–7,10,15,17,18,23]. In the present study, we showed that L^2 gene resistance-breaking tobamovirus has prevailed in major fields of green pepper in Japan (Table 1, Fig. 4). Moreover, the biological and serological properties and the nucleotide sequence of the coat protein gene showed that all field isolates were identical to a Japanese strain of PMMoV[10,15].

In this study, we also demonstrated that some cultivars of commercially available green pepper were unwillingly infected with PMMoV (Table 2, Fig. 6). Furthermore, the infectivity on *N. glutinosa* and green pepper showed that most viruses still retain their infectivity. Tobamovirus is the only virus that was proved to be transmitted to seedlings from the seed coat at germination[12,13]. In pepper plants, the frequency of seed transmission has been reported to range from very few to 30 %[20]. In a separate study, it

Table 2   Detection of PMMoV in commercial seeds of green pepper

Cultivars[a]	Virus detection by RT-PCR[c]	Infectivity on *N. glutinosa*[b]
A	+	−
B	+	+
C	−	−
D	−	−
E	−	−
F	−	−
G	+	+
H	+	+
I	+	+
J	−	−
K	+	+
L	−	−
M	−	−
N	−	−
O	−	−
P	−	−
Q	+	−
R	−	−
S	−	−
T	+	+

[a] Twenty cultivars were purchased from Japanese seed companies.

[b] RNA extracts from respective cultivars were assayed for the presence of PMMoV by RT-PCR. +, Detected; −, Not detected.

[c] Ten seeds were homogenized with 0.1M phosphate buffer (0.1 ml per seed) and the extracts were inoculated on the leaves of *N. glutinosa*. Necrotic local lesions developed on the leaves were observed 3 days after inoculation.

+, Detected; −, Not detected.

was also shown that transplanting of seedlings could result in nearly 100% infection, although only a few seedlings became infected when left undisturbed[9]. In 1978, an outbreak of tobamovirus-caused mosaic disease was reported in protected crops of green pepper at Nozaka, Chiba Prefecture, Japan[15]. According to the report by Nagai *et al.*[15], the circumstances were summarized as follows; i) the seedlings showed severe symptoms immediately after transplanting; ii)the incidence greatly increased to nearly 100% over the following 2 months. Based on these reports, the primary source of the viral infection may be related to dissemination of infected seeds and/or infected soil, since the virus is not dispersed by insect vectors[9,13].

Mink[13] emphasized that dissemination of infected seeds itself may provide a long-range dispersal of viruses, sometimes resulting in introduction of the virus into new fields as well as in increase of the virus concentration in soil[4,13]. In fact, the pepper seeds harvested from systemically infected plants were shown to retain the infectious virus, and the incidence of the virus infection in growing seeds was almost 100% (Figs. 4d and 6). Therefore, the insurance of virus-free seeds, including complete seed disinfection seems to be essential to reduce potential threats. Recently, we have developed a rapid and accurate immunostaining assay to detect PMMoV in harvested seeds of green pepper without detrimental effect on seed germination[19]. Thus, the RT-PCR technique presented here, together with the immunostaining assay[19], will be useful for diagnosis of infected seeds, including a seed disinfection before cultivation[21].

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