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VIRAL AND VIROID DISEASES

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Discrimination between tobamoviruses and their pathotypes for *L*-gene-mediated resistance in green pepper (*Capsicum annuum* L.) by reverse transcription-polymerase chain reaction

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Abstract A new method to discriminate between tobamoviruses and their pathotypes that infect green pepper (*Capsicum annuum* L.) was developed using reverse transcription-polymerase chain reaction (RT-PCR). The P_0 pathotype (*Tobacco mosaic virus*, *Tomato mosaic virus*, and *Tobacco mild green mosaic virus*) and the P_1 pathotype (*Paprika mild mottle virus*) were distinguished by RT-PCR using primers specific to each pathotype. However, the $P_{1,2}$ and $P_{1,2,3}$ pathotypes of *Pepper mild mottle virus* (PMMoV) could not be distinguished from each other using this procedure. The $P_{1,2}$ and $P_{1,2,3}$ pathotypes were differentiated by RT-nested PCR, in which a DNA fragment was first produced by RT-PCR using primers containing conserved sequences of PMMoV. The product was then used as the DNA template in a second PCR using primers specific to each pathotype. An immunocapture (IC) RT-PCR method was developed based on results from this study, which facilitated detection of tobamoviruses in pepper plants, seeds, and field soils and allowed the identification of their pathotypes.

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Introduction

Capsicum plants are one of the most popular vegetable crops in the world; however, diseases caused by tobamoviruses result in severe reduction in yield and quality, leading to serious economic loss (Alonso et al. 1989; Nagai et al. 1981). To protect *Capsicum* crops from tobamovirus diseases, tobamovirus-resistant cultivars have been bred (Barta et al. 1985; Matsumoto et al. 1999). In *Capsicum* species, four allelic genes, L^1, L^2, L^3, and L^4, are involved in resistance against tobamovirus infection (Boukema 1980, 1984). Tobamoviruses infecting *Capsicum* plants are classified into four pathotypes, P_0, P_1, $P_{1,2}$, and $P_{1,2,3}$, based on the infection patterns in *Capsicum* plants carrying the different *L* genes (Rast 1988). The coat protein (CP) of tobamoviruses has been shown to elicit L^2- or L^3-gene-mediated resistance (Berzal-Herranz et al. 1995; de la Cruz et al. 1997), and amino acid changes in the CP responsible for overcoming L^3-gene-mediated resistance have been identified (Berzal-Herranz et al. 1995; Hamada et al. 2002; Tsuda et al. 1998).

Five species of *Tobamovirus*, *Tobacco mosaic virus* (TMV, P_0), *Tomato mosaic virus* (ToMV, P_0), *Tobacco mild green mosaic virus* (TMGMV, P_0), *Paprika mild mottle virus* (PaMMV, P_1), and *Pepper mild mottle virus* (PMMoV, $P_{1,2}$ or $P_{1,2,3}$), have been reported as pathogens of *C. annuum* in Japan (Hamada et al. 2002, 2003; Nagai 1981; Nagai et al. 1981; Tsuda et al. 1998). Selection of cultivars resistant to the pathotype of tobamoviruses in the field is essential for the control of tobamovirus diseases. Therefore, it is important to identify the tobamovirus pathotypes occurring in fields. Inoculation tests using differential hosts or serological tests have conventionally been used to discriminate between pathotypes of tobamoviruses, but results from these tests are ambiguous. The use of reverse transcription-polymerase chain reaction (RT-PCR) (Letschert et al. 2002) or restriction fragment length polymorphism (RFLP) analysis

following RT-PCR (Velasco et al. 2002) successfully discriminated between the P_0 pathotypes (TMV, ToMV, and TMGMV), the $P_{1,2}$ pathotype of PMMoV, and the Italian type of $P_{1,2,3}$ PMMoV (Wetter et al. 1984; Berzal-Herranz et al. 1995). However, the P_1 pathotype, PaMMV, could not be distinguished by these methods. In addition to these PMMoVs, two other types of $P_{1,2,3}$ PMMoV, distinct from the Italian type, have been reported in Japan (Hamada et al. 2002; Tsuda et al. 1998). Here, we report an RT-PCR procedure for discriminating between the different pathotypes of tobamoviruses that infect *Capsicum* plants. Based on the results, we further developed a new method to detect and to discriminate between pathotypes of tobamoviruses using immunocapture (IC) RT-PCR.

Materials and methods

Viruses

Purified virions of TMV-OM (P_0) and ToMV-T (P_0) were provided by the National Agriculture Research Center. PMMoV-Pe1 ($P_{1,2}$) was isolated from a naturally infected green pepper plant from Kochi Prefecture, Japan. Purified virions of PMMoV-Pe1, TMGMV-J (P_0, Morishima et al. 2003), PaMMV-J (P_1, Hamada et al. 2003), PMMoV-Ge1, PMMoV-Tosa, and PMMoV-Oh (Ij, Italian and Oh type of the $P_{1,2,3}$, respectively; Hamada et al. 2002) were prepared as described previously (Takeuchi et al. 1999). Viral RNA was extracted from purified virions as described previously (Allison et al. 1990). The nucleotide sequence of the CP gene of PMMoV-Pe1 (DDBJ accession no. AB119482) was determined as described previously (Hamada et al. 2002).

cDNA clones

The PMMoV-J infectious cDNA clone, pTPW1 (Kirita et al. 1997), was kindly provided by Dr. S. Tsuda (National Agricultural Research Center). CP gene cDNA clones from PMMoV-Ge1, PMMoV-Tosa, and PMMoV-Oh, pUC/Ge1-CP, pUC/Tosa-CP, and pUC/Oh-CP, were prepared as follows: cDNA for the CP gene of PMMoV-Ge1, PMMoV-Tosa, and PMMoV-Oh was amplified by PCR using the primer pair PMF1/Bam-PMR1/Eco as described previously (Hamada et al. 2002). The products were digested with *Bam*H I and *Eco*R I (Takara, Ohtsu, Japan), and then ligated into the corresponding sites of pUC118 (Takara).

Discrimination between the P_0 and P_1 pathotypes

Three pairs of primers, TMF-TMR, LF-LR, and TGF-TGR, were prepared based on the published sequences of TMV-vulgare (Goelet et al. 1982), ToMV-L (Ohno et al. 1984), and TMGMV-J (Morishima et al. 2003) for detection of the P_0 pathotype using RT-PCR (Table 1). A pair of primers, P1F and P1R (Hamada et al. 2003), was used for detection of PaMMV by RT-PCR. RT-PCR was performed using the RNA PCR kit (AMV) Ver. 2.1 (Takara) with a PCR Thermal Cycler MP (Takara). To prevent nonspecific DNA amplification, random nonamers (Takara) were used as the primers for reverse transcription. The cDNA was synthesized from viral RNA using conditions of 30°C for 10 min, 42°C for 30 min and then 99°C for 5 min. PCR was performed with each set of primers using a 3-min preincubation at 94°C, 25 cycles of 30 s each of 94°C, 55°C, and 72°C, followed by 7 min at 72°C. After the PCR, 5 μl of each product was electrophoresed on a 1% agarose gel, and DNA fragments were detected by ethidium bromide staining.

Discrimination between the $P_{1,2}$ and $P_{1,2,3}$ pathotypes

Seven primers, JF, GF, TF, OF, J2F, R2, and TR, were designed based on the sequences of PMMoV-J (Kirita et al. 1997), PMMoV-Ge1, PMMoV-Tosa, and PMMoV-Oh (Fig. 1). The first and/or second bases in the 3′-terminus of sense or antisense primers corresponded to the mutation points in the viral RNA that characterize the pathotype of each isolate. Combinations of JF and R2, GF and R2, TF and TR, and OF and R2 were used for detection of the $P_{1,2}$ pathotype, Ij type, Italian type, and Oh type of $P_{1,2,3}$, respectively. A pair of primers, J2F and R2, was used for confirmation of the coexistence of the $P_{1,2}$ pathotype in the Oh type of $P_{1,2,3}$. A DNA fragment corresponding to the entire CP gene of PMMoV was first produced from viral RNA by RT-PCR with the primer pair PMF1-PMR1 (Hamada et al. 2002) using the same procedure as for discriminating between the P_0 and P_1 pathotypes. The product of RT-PCR at a final concentration of 10 fg/μl was used as a DNA template

Table 1. Oligonucleotide primers used to detect the P_0 pathotype of *tobamoviruses*

Target virus	Primer	Sequence	Position[a]	Size of products (bp)
TMV	TMF	5′-AAGTTTCGATCTCGAACCGG-3′	5527-5546	683
	TMR	5′-TTATGCATCTTGACTACCTC-3′	6190-6209	
ToMV	LF	5′-AGGGGCGTTCAGGCGGAAGG-3′	5585-5604	625
	LR	5′-AAACTTTATATTTCAGCACC-3′	6190-6209	
TMGMV	TGF	5′-GATGAAGTACCAATGGCTGTG-3′	5484-5504	728
	TGR	5′-TTGGTATCAGCCACCCTGAA-3′	6192-6211	

TMV, *Tobacco mosaic virus*; ToMV, *Tomato mosaic virus*; TMGMV, *Tobacco mild green mosaic virus*
[a] Position from the 5′ terminus of viral genomic RNA

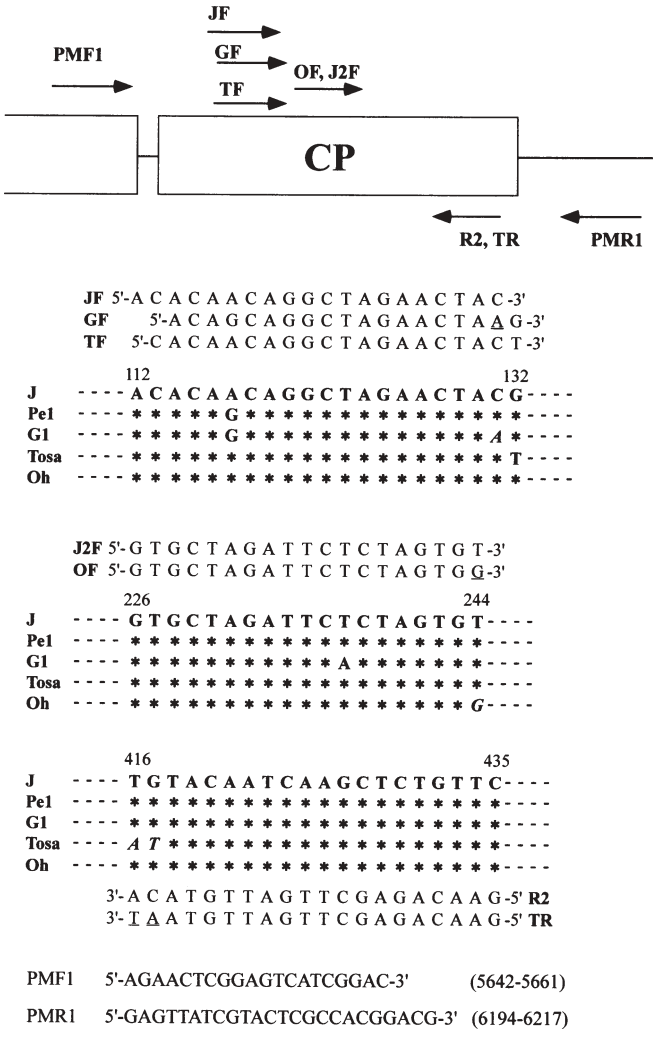

Fig. 1. Nucleotide sequence of five *Pepper mild mottle virus* (PMMoV) isolates and the oligonucleotide primers used to discriminate between the $P_{1,2}$ and $P_{1,2,3}$ pathotypes of PMMoV by reverse transcription-polymerase clain reaction (RT-PCR). The primer pairs JF-R2, GF-R2, TF-TR, and OF-R2 were used to detect the $P_{1,2}$ pathotype and the Ij, Italian, and Oh type of the $P_{1,2,3}$ pathotype, respectively. J2F and R2 were used for detection of the $P_{1,2}$ pathotype coexisting with the Oh type of the $P_{1,2,3}$ pathotype. *Numbers* above the nucleotide sequences indicate the position from the 5′-terminus of the coat protein (CP) gene. Identical sequences are indicated with *asterisks*. *Italics* indicate the mutation points characterizing the pathotype of each isolate, and *underlines* indicate the bases corresponding or complementary to the mutation points in each primer. PMF1 and PMR1 are the primers used to amplify the CP gene region of PMMoV, and numbers in parentheses indicate the nucleotide positions from the 5′-terminus of genomic RNA

for the second PCR to differentiate between the $P_{1,2}$ and $P_{1,2,3}$ pathotypes. The second PCR was performed with a 3-min preincubation at 94°, 25 cycles of 30 s each of 94°C, 60°C, and 72°C, followed by 7 min at 72°C, in a mixture containing 2.5 units *Ex Taq* DNA polymerase (Takara), *Ex Taq* buffer, 80 μM of each dNTP, and 0.4 μM each of the sense and antisense primers.

Immunocapture RT-PCR

IC-RT-PCR was performed as described by Jansen et al. (1990), with some modifications. PCR tubes were precoated with antibody against TMGMV-J by incubation with 20 μl of the antibody solution (40 μg/ml in 0.05 M carbonate buffer, pH 9.6) at 37°C for 2 h followed by three washes with phosphate-buffered saline containing 0.5% Tween 20 (PBST). Samples for IC-RT-PCR were prepared as follows. For plant tissues, approximately 0.1 g of tissue was ground in 10 ml of PBST using a mortar and pestle, and the homogenate was used further. For pepper seeds, ten seeds were placed in a microtube with 200 μl of PBST, shaken for 10 min, and the resultant supernatant was used for testing. For field soils, 1 g of soil was placed in a microtube with 800 μl of PBST and shaken for 30 min. The solution was clarified by centrifugation at 20,000 *g* for 10 min, and the supernatant was used further. Twenty microliters of tissue homogenates or extracts from seed or soil were kept overnight at 4°C in the precoated tube. After three washes with PBST, all components of the reverse transcription reaction except reverse transcriptase were added to the tube and heated to 80°C for 5 min. After the mixture cooled down, reverse transcriptase was added, and the reverse transcription reaction followed by PCR was performed as described earlier.

Preparation of pepper plants, seeds, and soils infested with tobamoviruses

Seedlings of green pepper were inoculated with purified virions of TMV-OM, ToMV-T, TMGMV-J, PaMMV-J, and four isolates of PMMoV, including Pe1, Ge1, Tosa, and Oh. Uninoculated upper leaves were harvested for detection of virus 8 days after inoculation. Naturally infected plants were also used. Green pepper plants showing mosaic or necrotic symptoms were collected from fields in Kochi Prefecture. Infection with tobamoviruses was confirmed by enzyme-linked immunosorbent assay (ELISA) (Suzuki et al. 1990) using antibody raised against TMGMV-J.

To prepare tobamovirus-infested seeds, green pepper plants were inoculated with purified virions of TMGMV-J, PaMMV-J, PMMoV-Pe1, PMMoV-Ge1, PMMoV-Tosa, and a mixture of PMMoV-Ge1 and PMMoV-Oh virions. Seeds were harvested from these plants, and infestation with tobamovirus was confirmed by direct immunostaining assay (DISA, Takeuchi et al. 1999) for 10 to 19 seeds from each treatment. Another ten seeds from each treatment were used for IC-RT-PCR. Commercial seeds of four green pepper cultivars were also used. Ten seeds from each cultivar were used for IC-RT-PCR, and another 16 to 19 seeds were subjected to DISA.

To prepare tobamovirus-infested soil, green pepper plants grown in commercial compost (Tsuchitaro, Sumirin Agro-products, Aichi, Japan) were inoculated with purified virions of PMMoV-Tosa. After cultivation for 5 months, the soil under the pepper plants was recovered. Infestation with tobamovirus was confirmed by indirect (Id) ELISA

(Takeuchi et al. 2000) using 0.3 g of soil. IC-RT-PCR was performed on the samples using another 1 g of soil. Soil collected from four different pepper fields in Kochi Prefecture (Gray Lowland soil) was also analyzed. Surface soil under the pepper plants was collected from one randomly selected point in each field. One gram of each soil sample was used for IC-RT-PCR, and another 0.3 g was subjected to Id-ELISA.

Inoculation tests

Capsicum annuum cv. Shosuke (L^+/L^+), *C. annuum* cv. Verbeterde Glas (L^1/L^1), *C. frutescens* cv. Tabasco (L^2/L^2), *C. chinense* PI159236 (L^3/L^3), *C. chacoense* PI260429 (L^4/L^4), *Lycopersicon esculentum* cv. Ohgata Fukuju, and *Nicotiana sylvestris* were used as differential hosts. All plants were grown in plastic pots containing commercial compost (Tsuchitaro) in a greenhouse. Differential host plants were mechanically inoculated with crude sap of infected tissue prepared in 0.1 M phosphate buffer, pH 7.0. Three plants of each differential host were used in each inoculation test. Infection with tobamovirus was confirmed by ELISA 7 days after inoculation for inoculated leaves and 14 days after inoculation for uninoculated upper leaves.

Results and discussion

Discrimination between P_0 and P_1 pathotypes

The four pairs of primers (TMF-TMR, LF-LR, TGF-TGR, and P1F-P1R) for the RT-PCR specifically amplified cDNA generated from the viral RNA of TMV, ToMV, TMGMV, and PaMMV, respectively, and produced a single DNA fragment (Fig. 2). This indicates that these four tobamoviruses can be distinguished from each other and that the P_1 pathotype, PaMMV, can be discriminated from the P_0 tobamoviruses by this procedure.

Discrimination between $P_{1,2}$ and $P_{1,2,3}$ pathotypes

When RT-PCR was performed using the same procedure and conditions for discriminating between the P_0 and P_1 pathotypes, the primer pair TF-TR specifically amplified cDNA generated from the target viral RNA of PMMoV-Tosa. However, the primer pair JF-R2, which was expected to work only with the viral RNA from Pe1 and Oh, also amplified a cDNA product from Ge1. Similarly, the primer pair GF-R2 amplified cDNA not only from Ge1 but also from Pe1 and Oh. The primer pair OF-R2 amplified cDNA generated from not only Oh but also Tosa. Thus, the procedures and conditions used in the RT-PCR were not sufficient to discriminate between the $P_{1,2}$ and $P_{1,2,3}$ pathotypes because of nonspecific DNA amplification.

To prevent nonspecific reactions, PCR conditions were examined using cDNA clones as templates. It was found that nonspecific DNA fragments were amplified when the concentration of template DNA was higher than 1 pg/μl or the annealing temperature was below 60°C (data not shown). Based on these results, a nested PCR method was devised. RT-PCR using a PMMoV-specific primer pair, PMF1-PMR1 (Hamada et al. 2002), was first performed under the same conditions used to differentiate P_0 and P_1. In this RT-PCR, DNA fragments corresponding to the whole region of the CP gene were amplified specifically from PMMoV regardless of their pathotype. The concentration of the resulting PCR product was estimated by comparing the intensity of ethidium bromide staining after agarose gel electrophoresis to a known amount of *Hin*d III-digested λ DNA. The product, at a final concentration of approximately 10 fg/μl, was added to the reaction mixture for the second PCR. Temperature for annealing in the second PCR was adjusted to 60°C. After the RT-nested PCR, DNA fragments were specifically produced from the corresponding type of PMMoV (Fig. 3). PMMoV-Oh contains the same nucleotide sequences as PMMoV-J at the target points of the primers JF and R2, 112–131 bases and 416–435 bases from the 5′-terminus of the CP gene, respectively

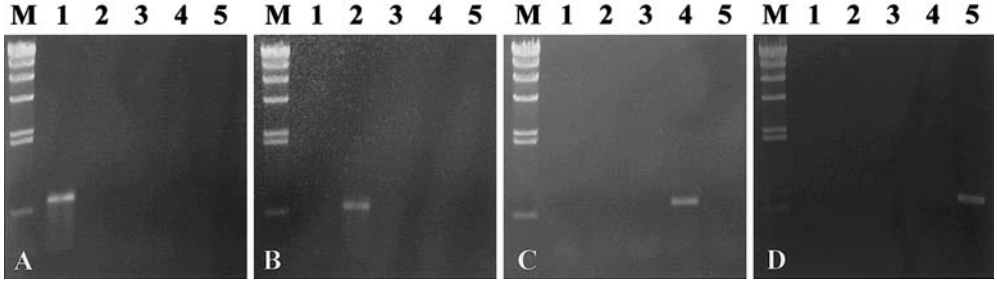

Fig. 2. Discrimination between the P_0 and P_1 pathotypes of *tobamoviruses* by RT-PCR. Primer pairs TMF-TMR specific to *Tobacco mosaic virus* (TMV) (**A**), LF-LR specific to *Tomato mosaic virus* (ToMV) (**B**), TGF-TGR specific to *Tobacco mild green mosaic virus* (TMGMV) (**C**), and P1F-P1R specific to *Paprika mild mottle virus* (PaMMV) (**D**) were used. Genomic RNA extracted from purified virions of TMV-OM (*lane 1*), ToMV-T (*lane 2*), PMMoV-Pe1 (*lane 3*), TMGMV-J (*lane 4*), and PaMMV-J (*lane 5*) were used as templates for RT-PCR. *M*, lambda DNA digested with *Hin*d III

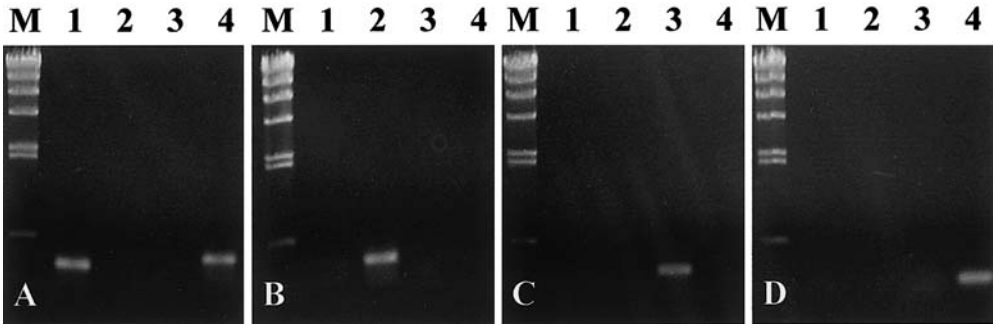

Fig. 3. Discrimination between the $P_{1,2}$ pathotype and three types of the $P_{1,2,3}$ pathotype of PMMoV by RT-nested PCR. Primer pair JF-R2 specific to the $P_{1,2}$ pathotype and Oh type of $P_{1,2,3}$ pathotype (**A**), GF-R2 specific to the Ij-type of $P_{1,2,3}$ (**B**), TF-TR specific to Italian-type of $P_{1,2,3}$ (**C**), and OF-R2 specific to Oh type of $P_{1,2,3}$ (**D**) were used for RT-nested PCR. The RT-PCR products amplified from viral RNA of PMMoV-Pe1 (*lane 1*), Ge1 (*lane 2*), Tosa (*lane 3*), and Oh (*lane 4*) with PMF1 and PMR1 primers were used as template DNA. *M*, lambda DNA digested with *Hin*d III

(Fig. 1). Therefore, the primer pair JF-R2 amplified DNA derived from PMMoV-Oh as well as from PMMoV-Pe1, the $P_{1,2}$ pathotype (Fig. 3A). This indicates that mixtures of both the Oh type of the $P_{1,2,3}$ pathotype and a $P_{1,2}$ pathotype such as Pe1 will result in the same DNA amplification pattern as found with the Oh type alone. For practical use, such as field diagnosis to select appropriate cultivars, determining the occurrence of a $P_{1,2,3}$ pathotype such as Oh is very important, and coexistence of the $P_{1,2}$ pathotype with the Oh type may be of negligible importance because we can use cultivars carrying the L^4 gene based on the occurrence of the $P_{1,2,3}$ pathotype, which are resistant to the P_0, P_1, and $P_{1,2}$ pathotypes as well as to the $P_{1,2,3}$ pathotype. On the other hand, it is still important in epidemiological research to detect the $P_{1,2}$ pathotype coexisting with the Oh type, because such research requires detailed information about the distribution of each pathotype. To clarify whether DNA amplification by the primers JF-R2 and OF-R2 indicated the existence of the Oh type alone or the coexistence of the Oh type and the $P_{1,2}$ pathotype, an additional pair of primers, J2F-R2, was used for the second PCR. Viral RNA was extracted from purified virions of PMMoV-Oh and from a mixture of PMMoV-Oh and PMMoV-Pe1 virions and subjected to the RT-nested PCR. For primer pairs JF-R2, GF-R2, TF-TR, and OF-R2, DNA amplification patterns were identical for Oh alone and for the mixture of Oh and Pe1 (Fig. 4, lanes 1–4). However, a DNA fragment indicating the $P_{1,2}$ pathotype was produced from the mixture of Oh and Pe1 but not from Oh alone by the J2F-R2 primers (Fig. 4, lane 5). Thus, coexistence of the $P_{1,2}$ pathotype with the Oh type can be detected by the additional use of the J2F-R2 primers if needed.

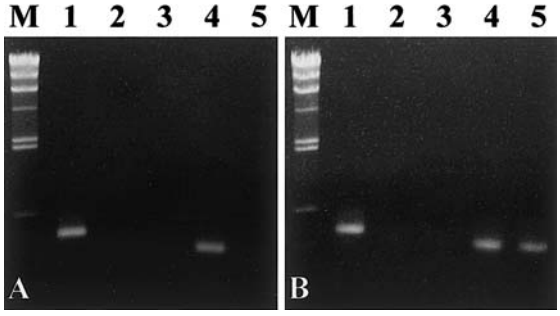

Fig. 4. Discrimination of the Oh type of the $P_{1,2,3}$ pathotype from a mixture of Oh type and the $P_{1,2}$ pathotype by RT-nested PCR. DNA fragments were produced from viral RNA of PMMoV-Oh and the mixture of PMMoV-Oh and PMMoV-Pe1 by RT-PCR using primers PMF1 and PMR1. The DNA fragments derived from PMMoV- Oh (**A**) or the mixture of PMMoV-Oh and PMMoV-Pe1 (**B**) were used as the template for the second PCR. Five pairs of primers, JF-R2 (*lane 1*), GF-R2 (*lane 2*), TF-TR (*lane 3*), OF-R2 (*lane 4*), and J2F-R2 (*lane 5*) were used for the second PCR. *M*, lambda DNA digested with *Hin*d III

Detection of tobamoviruses from pepper leaves, seeds, and soils, and identification of their pathotypes

To test whether the RT-PCR method could be used to detect tobamoviruses in pepper leaves, seeds, and soils, and to identify the pathotypes, the RT-PCR method was also used with artificially inoculated pepper plants, as well as with infested seeds and soils. To simplify the RNA extraction and to test large numbers of samples at the same time, an IC-RT-PCR procedure was used. When leaves of inoculated pepper plants were subjected to IC-RT-PCR, the tobamovirus used as the inoculum was detected and identified (data not shown). The same kind of tobamovirus used for inoculating the mother plant was detected in infested seeds (Fig. 5). In soils, no DNA fragments were generated by the IC-RT-PCR method, although infestation with PMMoV was confirmed by Id-ELISA (absorbance of 0.695 at 405 nm). Therefore, the first PCR was changed to 35 cycles of 60 s each of 94°C, 55°C, and 72°C, followed by 7 min at 72°C. Under the improved conditions, PMMoV was detected from infested soil using IC-RT-PCR (Fig. 5).

Detection of tobamoviruses from field plants, commercial seeds and field soils, and identification of the pathotypes

To confirm the practical use of the IC-RT-PCR procedure, field samples of diseased pepper plants were subjected to

IC-RT-PCR. At the same time, the results of inoculation tests using differential hosts were compared. Typical results are summarized in Fig. 6 and Table 2. Inoculation tests of plant samples, which were assessed by IC-RT-PCR to be infected with either TMV, TMGMV, PaMMV, the $P_{1,2}$ pathotype of PMMoV, or the Oh type of PMMoV alone, showed infection patterns specific to each virus (sample nos. 6, 7, 8, 1, and 4 in Fig. 6 and Table 2). Similarly, pathotype-specific discrimination was obtained for Ij or Italian type of PMMoV in the inoculation tests, although they could not be distinguished from each other by the inoculation tests (sample nos. 2 and 3 in Fig. 6 and Table 2). In plant samples determined to be doubly infected with TMV and TMGMV by IC-RT-PCR, infection patterns in differential hosts showed the presence of two different tobamoviruses, in this case TMV and TMGMV (sample no. 9 in Fig. 6 and Table 2). In the case of a plant sample shown to be triply infected with TMV, TMGMV, and the $P_{1,2}$ pathotype of PMMoV by IC-RT-PCR, inoculation tests failed to show whether the plant was infected with TMGMV (sample no. 5 in Fig. 6 and Table 2), because triple infection with TMV, TMGMV, and the $P_{1,2}$ pathotype of PMMoV showed the same infection pattern as double infection with TMV and the $P_{1,2}$ pathotype of PMMoV. To clarify whether the plant sample was really infected with three tobamoviruses, *N. tabacum* cv. Xanthi-nc plants were inoculated with the crude sap of the original pepper plant. In 32 local lesions produced in the inoculated leaves, tobamoviruses were discriminated by IC-RT-PCR. Single infection by TMV and PMMoV was detected in 7 and 22 local lesions, respectively. Single infection by TMGMV was found in one local lesion and double infection by PMMoV and TMGMV in two local lesions. Two local lesions with TMV, two with PMMoV, and one with TMGMV were selected randomly, and each infecting virus was propagated in *N. benthamiana* plants for inoculation tests. In the inoculation tests, infection patterns corresponding to each virus indicated by the IC-RT-PCR were observed (data not shown). Thus, the IC-RT-PCR is useful for detecting tobamoviruses in pepper plants and determining the pathotype, especially for tissues infected with two or three different tobamoviruses that are not easily discriminated by inoculation tests.

To further test the utility of the IC-RT-PCR in practical use, commercial pepper seeds and field soils were subjected to IC-RT-PCR, and the results were compared with those of DISA or Id-ELISA. Seeds of four commercial cultivars were used. Seeds from three cultivars were stained in DISA

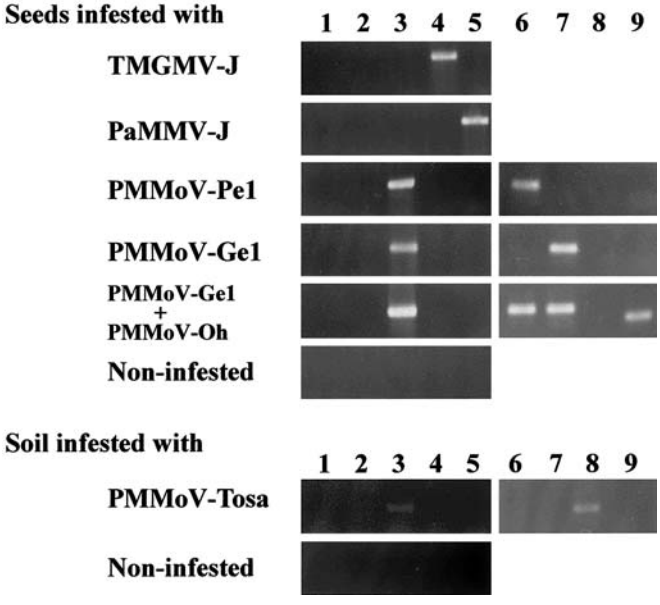

Fig. 5. Detection of tobamoviruses from pepper seeds and soils and identification of their pathotypes. Tobamovirus-infested and noninfested pepper seeds or soils were shaken in phosphate-buffered saline with Tween 20 (PBST), and the supernatants were subjected to immunocapture (IC)-RT-PCR using the primer pairs TMF-TMR (*lane 1*), LF-LR (*lane 2*), PMF1-PMR1 (*lane 3*), TGF-TGR (*lane 4*), P1F-P1R (*lane 5*), JF-R2 (*lane 6*), GF-R2 (*lane 7*), TF-TR (*lane 8*), and OF-R2 (lane 9)

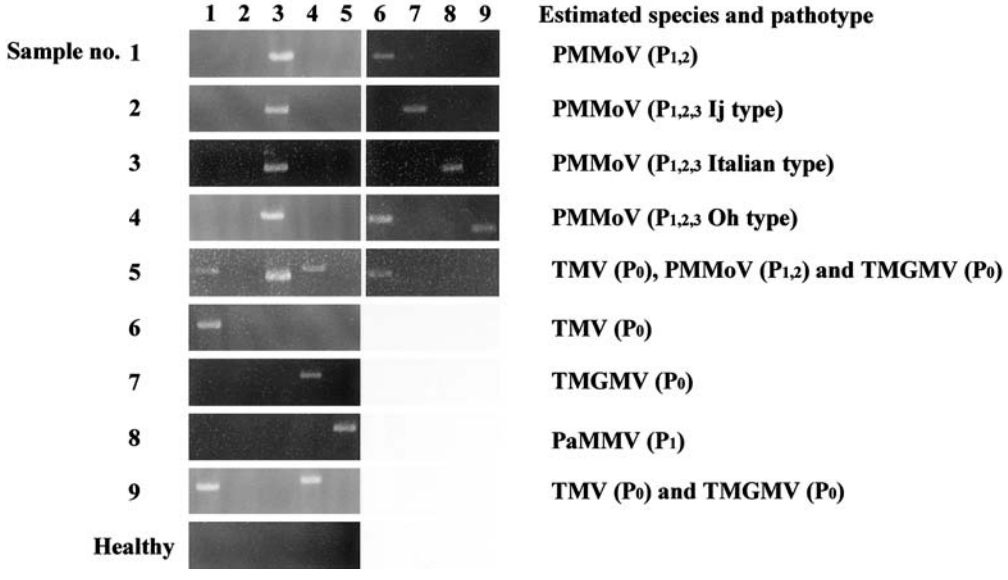

Fig. 6. Detection of tobamoviruses from naturally infected green pepper plants collected from fields and identification of their pathotypes. Leaf homogenates in PBST were subjected to IC-RT-PCR using the primer pairs TMF-TMR (*lane 1*), LF-LR (*lane 2*), PMF1-PMR1 (*lane 3*), TGF-TGR (*lane 4*), P1F-P1R (*lane 5*), JF-R2 (*lane 6*), GF-R2 (*lane 7*), TF-TR (*lane 8*), and OF-R2 (*lane 9*). Sample nos. 1 to 9 correspond to those in Table 2

Table 2. Infection patterns of tobamoviruses detected from naturally infected green pepper plants in several differential hosts

Sampel no.[a]	Species and pathotype estimated by IC-RT-PCR	*Capsicum annuum* cv. Shosuke (L^+/L^+)	*C. annuum* cv. Verbeterde Glas (L^1/L^1)	*C. frutescens* cv. Tabasco (L^2/L^2)	*C. chinense* PI159236 (L^3/L^3)	*C. chacoense* PI260429 (L^4/L^4)	*Lycopersicon esculentum* cv. Ohgata Fukuju	*Nicotiana sylvestris*
1	PMMoV ($P_{1,2}$)	M[b]	M	M	L	L	–	L
2	PMMoV ($P_{1,2,3}$ Ij type)	M	M	M	M	L	–	L
3	PMMoV ($P_{1,2,3}$ Italian type)	M	M	M	M	L	–	L
4	PMMoV ($P_{1,2,3}$ Oh type)	M	M	M	N	L	–	L
5	TMV (P_0), PMMoV ($P_{1,2}$) and TMGMV (P_0)	M	L and M	L and M	L	L	M	L and M
6	TMV (P_0)	M	L	L	L	L	M	M
7	TMGMV (P_0)	M	L	L	L	L	–	L
8	PaMMV (P_1)	M	M	L	L	L	+	L
9	TMV (P_0) and TMGMV (P_0)	M	L	L	L	L	M	L and M

Infection with tobamoviruses was confirmed by ELISA 7 d and 14 d after inoculation for inoculated leaves and uninoculated upper leaves, respectively
M, systemic infection with mosaic; N, systemic infection with necrosis; L, local infection with necrotic spots; L and S, necrotic spots in inoculated leaves and systemic mosaic; +, symptomless infection; –, no infection; IC-RT-PCR, immunocapture-reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; PMMoV, *Pepper mild mottle virus*; PaMMV, *Paprika mild mottle virus*

[a] Sample nos. 1 to 9 correspond to those in Fig. 6

Table 3. Detection of tobamoviruses from commercial pepper seeds and identification of their pathotype by IC-RT-PCR and by direct immunostaining assay (DISA)

Cultivar	Tobamovirus detected by IC-RT-PCR	Stain in DISA
A	TMGMV	+[a]
B	PMMoV $P_{1,2}$	+
C	PMMoV $P_{1,2}$ and TMGMV	+
D	Not detected	–

[a] Pepper seeds were stained (+) or not (–) after DISA treatment

Table 4. Detection of tobamoviruses from field soils and identification of their pathotype

Field	Tobamovirus detected by IC-RT-PCR	A_{405} in ELISA
1	PMMoV $P_{1,2,3}$ Oh type	0.053[a]
2	PMMoV $P_{1,2}$	0.000
3	PMMoV $P_{1,2,3}$ Oh type	0.180
4	Not detected	0.000

[a] Absorbance at 405 nm was measured after 1 h of incubation with substrate at 25°C. Figures are the mean of three wells

but not the fourth (Table 3). Analysis by the IC-RT-PCR gave similar results. The viruses detected from these three cultivars were predicted to be TMGMV, $P_{1,2}$ of PMMoV, and a combination of both (Table 3). These results suggest that the use of IC-RT-PCR facilitates detection of tobamoviruses and identification of their pathotypes in pepper seeds.

Field soils collected from four different areas were tested for tobamoviruses using the IC-RT-PCR method. Results showed that the $P_{1,2}$ or the $P_{1,2,3}$ Oh type of PMMoV was clearly detected in three of four field soil samples (Table 4), although tests of the samples by ELISA failed to detect virus in one sample and the absorbance values for the other two samples were very low. This suggests that the IC-RT-PCR method is very sensitive and useful for detection of tobamoviruses in soil and determining their pathotypes in pepper fields.

Tobamoviruses are seed-borne in green pepper (Tosic et al. 1980). Once the viruses are carried into fields with seeds, they spread quickly to healthy plants by mechanical transmission through cultivation and natural contact of the plants with the systemically infected plants grown from the infested seeds (Broadbent 1976). The viruses remain in the soil even after cultivation and serve as a primary source of infection in the next cultivation (Pares and Gunn 1989). Because we have no effective measures to prevent contact or soil transmission of tobamoviruses, the control of tobamoviruses in green pepper largely depends on detection and elimination of contaminated seeds before cultivation as well as use of resistant cultivars. We previously developed DISA and Id-ELISA techniques to detect tobamoviruses in seeds and soils. These methods are very useful for field surveys and have been used on many commercial seeds and field soils (unpublished data), but they are insufficient to identify the pathotypes of tobamoviruses. Therefore, the IC-RT-PCR method described here will

facilitate the detection of tobamoviruses and determination of their pathotypes from seeds and soils and will aid in the selection of appropriate cultivars and the prevention of the spread of tobamoviruses through seeds and soils. Taken together, the IC-RT-PCR method is a practical technique for detecting tobamoviruses and identifying their pathotypes not only in *Capsicum* leaves but also in seeds and field soils.

References

Allison RF, Thompson C, Ahlquist P (1990) Regeneration of a functional RNA virus genome by recombination between deletion mutants and requirement for cowpea chlorotic mottle virus 3a and coat genes for systemic infection. Proc Natl Acad Sci U S A 87: 1820–1824

Alonso E, Garcia Luque I, Avila-Rincon MJ, Wicke B, Serra MT, Diaz-Ruiz JR (1989) A tobamovirus causing heavy losses in protected pepper crops in Spain. J Phytopathol 125:67–76

Barta A, Niemi HAT, Salamon P (1985) Establishing TMV-resistant pepper varieties. Capsicum Newslett 4:49

Berzal-Herranz A, De La Cruz A, Tenllado F, Diaz-Ruiz JR, Lopez L, Sanz AI, Vaquero C, Serra MT, Garcia-Luque I (1995) The *Capsicum* L^3 gene-mediated resistance against the tobamoviruses is elicited by the coat protein. Virology 209:498–505

Boukema IW (1980) Allelism of genes controlling resistance to TMV in *Capsicum* L. Euphytica 29:433–439

Boukema IW (1984) Resistance to TMV in *Capsicum chacoense* Hunz. is governed by an allele of the L-locus. Capsicum Newslett 3:47–48

Broadbent L (1976) Epidemiology and control of tomato mosaic virus. Annu Rev Phytopathol 14:75–96

de la Cruz A, Lopez L, Tenllado F, Diaz-Ruiz JR, Sanz AI, Vaquero C, Serra MT, Garcia L (1997) The coat protein is required for the elicitation of the *Capsicum* L^2 gene-mediated resistance against the tobamoviruses. Mol Plan-Microbe Interact 10:107–113

Goelet P, Lomonossoff GP, Butler PJG, Akam ME, Gait MJ, Karn J (1982) Nucleotide sequence of tobacco mosaic virus RNA. Proc Natl Acad Sci U S A 79:5818–5822

Hamada H, Takeuchi S, Kiba A, Tsuda S, Hikichi Y, Okuno T (2002) Amino acid changes in *Pepper mild mottle virus* coat protein that affect L^3 gene-mediated resistance in pepper. J Gen Plant Pathol 68:155–162

Hamada H, Takeuchi S, Morita Y, Sawada H, Kiba A, Hikichi Y (2003) Characterization of *Paprika mild mottle virus* first isolated in Japan. J Gen Plant Pathol 69:199–204

Jansen RW, Siegl G, Lemon SM (1990) Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc Natl Acad Sci U S A 87:2867–2871

Kirita M, Akutsu K, Watanabe Y, Tsuda S (1997) Nucleotide sequence of the Japanese isolate of pepper mild mottle tobamovirus (TMV-P) RNA. Ann Phytopathol Soc Jpn 63:373–376

Letschert B, Adam G, Lesemann DE, Willingmann P, Heinze C (2002) Detection and differentiation of serologically cross-reacting tobamoviruses of economical importance by RT-PCR and RT-PCR-RFLP. J Virol Methods 106:1–10

Matsumoto M, Nitta M, Sawada H, Nomachi A (1999) "Tosahime R", a PMMoV resistant sweet pepper (in Japanese with English summary) Bull Kochi Agric Res Cent 8:47–52

Morishima N, Ido T, Hamada H, Yoshimoto E, Mizumoto H, Takeuchi S, Kiba A, Hikichi Y, Okuno T (2003) Infectious in vitro transcripts from a cDNA clone of *Tobacco mild green mosaic tobamovirus* and its biological activity in host and nonhost plants and in their protoplasts. J Gen Plant Pathol 69:335–338

Nagai Y (1981) Control of mosaic disease of tomato and sweet pepper caused by tobacco mosaic virus (in Japanese with English summary). Spec Bull Chiba Agric Exp Stn 9:1–109

Nagai Y, Takeuchi T, Tochihara H (1981) A new mosaic disease of sweet pepper caused by pepper strain of tobacco mosaic virus. Ann Phytopathol Soc Jpn 47:541–546

Ohno T, Aoyagi M, Yamanashi Y, Saito H, Ikawa S, Meshi T, Okada Y (1984) Nucleotide sequence of the tobacco mosaic virus (tomato strain) genome and comparison with the common strain genome. J Biochem 96:1915–1923

Pares RD, Gunn LV (1989) The role of non-vectored soil transmission as a primary source of infection by pepper mild mottle and cucumber mosaic viruses in glasshouse-grown *Capsicum* in Australia. J Phytopathol 126:353–360

Rast ATB (1988) Pepper tobamoviruses and pathotypes used in resistance breeding. Capsicum Newslett 7:20–23

Suzuki N, Shirako Y, Ehara Y (1990) A simple method of elimination of non-specific reactions in non-precoated indirect and electroblot enzyme-linked immunosorbent assay procedures used for detection of zucchini yellow mosaic virus. Ann Phytopathol Soc Jpn 56:337–341

Takeuchi S, Hikichi Y, Kawada Y, Okuno T (1999) Direct immunostaining assay, a new simplified technique for detection of tobamoviruses from seeds of green pepper (*Capsicum annuum* L.). Ann Phytopathol Soc Jpn 65:189–191

Takeuchi S, Hikichi Y, Kawada Y, Okuno T (2000) Detection of tobamoviruses from soils by non-precoated indirect ELISA. J Gen Plant Pathol 66:153–158

Tosic M, Sutic D, Pesic Z (1980) Transmission of tobacco mosaic virus through pepper (*Capsicum annuum* L.) seed. Phytopath Z 97:10–13

Tsuda S, Kirita M, Watanabe Y (1998) Characterization of a pepper mild mottle tobamovirus strain capable of overcoming the L^3 gene-mediated resistance, distinct from the resistance-breaking Italian isolate. Mol Plant-Microbe Interact 11:327–331

Velasco L, Janssen D, Ruiz-Garcia L, Segundo E, Cuadrado IM (2002) The complete nucleotide sequence and development of a differential detection assay for a pepper mild mottle virus (PMMoV) isolate that overcomes L3 resistance in pepper. J Virol Methods 106:135–140

Wetter C, Conti M, Altschuh D, Tabillion R, van Regenmortel MHV (1984) Pepper mild mottle virus, a tobamovirus infecting pepper cultivars in Sicily. Phytopathology 74:405–410