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Efficient Inactivation of *Pepper Mild Mottle Virus* (PMMoV) in Harvested Seeds of Green Pepper (*Capsicum annuum* L.) Assessed by a Reverse Transcription and Polymerase Chain Reaction (RT-PCR)-based Amplification

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A method for efficient inactivation of *Pepper mild mottle virus* (PMMoV) in harvested seeds of green pepper was examined based on the infectivity on the leaves of *Nicotiana glutinosa* L. (a local lesion host) and a reverse transcription and polymerase chain reaction (RT-PCR)-based amplification of the viral RNA. The seed homogenates from PMMoV-infected plants produced a large number of necrotic local lesion in *N. glutinosa*, but soaking the seeds in 10% (w/v) *tri*-sodium phosphate (Na_3PO_4) for 20 min or dry sterilization (70°C, 3 h) effectively eliminated the abundance of PMMoV. However, no necrotic lesion on *N. glutinosa* or the RT-PCR-based amplification was observed with seeds that had been disinfected by Na_3PO_4 in combination with dry sterilization.

Key words : *Capsicum annuum* L., *Pepper mild mottle virus* (PMMoV), RT-PCR, seed disinfection, seed transmission

Introdaction

Pepper mild mottle virus (PMMoV; formerly referred to as *Tobacco mosaic virus* pepper strain, TMV-P[9]) is the virus so far described as infecting resistant cultivars conferred by *L* genes of *Capsicum* plants[1]. PMMoV is now widely distributed in Japan[5,8-13], and the virus infection often causes yield loss in pepper crops[8,9,13]. Although the dispersal of this tobamovirus is assisted most commonly by contact transmission, sometimes causing whole fields to be abandoned[4], the major source of the primary infection is thought to result from soil transmission during transplanting or seed transmission[4]. In our previous study[12], we have shown that about 40% of cultivars of commercially available green pepper had been unwillingly infected with PMMoV. Tosic *et al.*[11] reported that about 30% of pepper plant seedlings became infected through infected seeds. Therefore, the insurance of virus-free seeds including complete seed disinfection is essential for reducing potent threats, since dissemination of infected seeds may often contribute to a long-range dispersal of viruses, sometimes resulting in the introduction of viruses into new fields[6,7]. In this communication, we report an improved method for disinfection of PMMoV-infected seeds assessed by an RT-PCR that allows detection of the virus with an increased sensitivity[12].

Materials and Methods

Five week-old seedlings of green pepper (*Capsicum annuum* L.) cv. Shosuke were mechanically inoculated with a PMMoV I-6, originally isolated in Iwate Prefecture in 1996[12], and the seeds were harvested from the growing fruits at 2 months after inoculation. The incidence of virus infection in individual seeds

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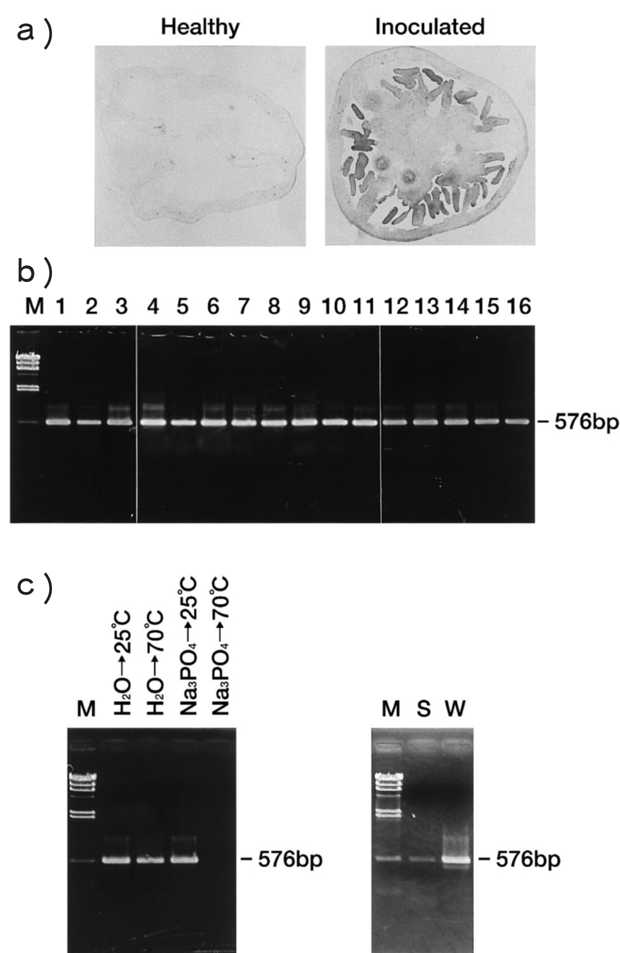

Fig. 1 Preparation of infected seeds of green pepper and their disinfection assessed by an RT-PCR analysis. The seeds were harvested from the growing fruits at 2 months after inoculation and the virus infection was first checked by a tissue printing immunoassay with PMMoV antiserum (a) and by an RT-PCR (b). The growing fruits harvested from systemically infected plant were cut into round slices and were pressed onto a polyvinylidene difluoride (PVDF) membrane. For RT-PCR analysis, RNA extracts from individual seeds were used for PCR amplification as described in the text. Lane M was λDNA digested with *Hind* III. Lanes 1-16 were a typical amplification of 16 individual seeds harvested from infected plants. c), Disinfection of PMMoV-infected seeds assessed by RT-PCR. RNA extracts from seeds that had been disinfected with 10% Na_3PO_4 and/or dry sterilization were assayed for the presence of PMMoV-RNA. Lane M was λDNA digested with *Hind* III. The right figure shows an incomplete disinfection by Na_3PO_4. After washing with 10% Na_3PO_4 for 20 min, RNAs were extracted from the seeds and were used for the RT-PCR. Five microliters of the washing solution was also assayed. Note that soaking the infected seeds in 10% Na_3PO_4 for 20 min could release PMMoV into the washing solution (lane W), but the viral RNA was still retained in the seeds (lane S).

was first checked by a tissue printing immunoassay with a PMMoV antiserum (Fig. 1a) and an RT-PCR analysis (Fig. 1b), as described previously[12]. For RT-PCR analysis, first-strand cDNA was synthesized from total RNAs of infected seeds with an avian myeloblastosis virus (AMV) reverse transcriptase (TaKaRa) using an oligonucleotide, 5'-GAG TTA TCG TAC TCG CCA CG-3', complementary to nucleotide position at 6198 to 6217 of PMMoV-RNA[12]. The synthesized cDNAs were mixed for specific amplification with another oligonucleotide, 5'-AGA ACT CGG AGT CAT CGG AC-3', corresponding to the nucleotide position at 5642 to 5661, in the reaction mixture (100 μl) 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). The PCR was done for 25 cycles with the program (94°C, 1 min; 45°C, 1 min; 72°C, 1 min) with a TaKaRa TP3000 thermal cycler. Our preliminary experiments showed that the RT-PCR assay allowed the amplification of a 576 bp

Table 1 Preparation of infected seeds from systemically infected plants and their determination of incidence for PMMoV infection as assessed by an RT-PCR

Numbers of seeds tested[a]	Numbers of experiments	Detection by RT-PCR[b]	Incidence[c]
30	2	+	2/2
20	3	+	3/3
10	5	+	5/5
5	5	+	5/5
1	10	+	10/10

[a]Seeds were harvested from systemically infected plants at 2 months after inoculation.
[b]RNAs were extracted from the seeds and were dissolved in 0.1 ml sterilized water. One microliter of the RNA extracts was used for the RT-PCR analysis. +, DNA fragment specific for PMMoV was observed.
[c]Repeated experiments showed that the RT-PCR was able to detect PMMoV-RNA even in the RNA extracts from single seeds.

DNA fragment specific for PMMoV even with RNA extracts from the single seeds (Table 1).

Results and Discussion

Figure 1b shows a typical amplification of seeds harvested from systemically infected plants. These results indicated that the incidence of virus infection was almost 100%. In addition, when the seed homogenates were rubbed on fully expanded leaves of *Nicotiana glutinosa* L., over 100 distinct necrotic local

lesions were produced within 2 days after inoculation (see Table 2). We therefore considered that seeds harvested from systemically infected plants retain the infectious virus. Such seeds (designated as "infected seeds" below) were routinely prepared from systemically infected plants and used throughout the experiments as described below.

When the infected seeds were homogenated and the extracts were inoculated on the leaves of *N. glutinosa*, a large number of necrotic local lesions were produced within 2 days after inoculation (Table 2). However, the homogenates from seeds that had been sterilized at 70°C for 3h produced small amounts of necrotic local lesions on leaves of *N. glutinosa*, compared to the untreated seeds (Table 2). Furthermore, soaking the infected seeds in 10% (w/v) Na_3PO_4 for 20 min reduced the numbers of necrotic local lesions on *N. glutinosa* almost completely (Table 2), indicating that Na_3PO_4 can effectively eliminate the virus which was retained on/in seed surface.

To further verify the efficacy of the respective treatments to reduce the virus, RNA extracts from the disinfected seeds were analyzed for the presence of PMMoV by the RT-PCR. As shown in Figure 1c, the DNA fragments (576 bp) specific for PMMoV were still found both in the seeds that had been disinfected by 10% Na_3PO_4 or by dry sterilization (70°C, 3h). This result indicated that neither treatment was not able to disinfect PMMoV entirely. However, when infected seeds were first treated with a solution of 10% Na_3PO_4 and then sterilized by heat treatment (70°C, 3h), no necrotic local lesion was observed on *N. glutinosa* (Table 2). Moreover, the RT-PCR analysis gave no distinct band specific for the virus (Fig. 1c). These results indicate that the combination of both treatments is more effective for disinfection of PMMoV-infected seeds. Such a treatment also enabled elimination of the virus in commercial seeds that had been infected with PMMoV without any detrimental effect on seed germination[10] (data not shown).

Tobamoviruses including PMMoV are known to be retained largely on the surface of the seed coat[4,6,7,10] and the viruses often cause infection during germination and/or handling when transplanting[6,7]. For this reason, disinfection of harvested pepper crop seeds has been commonly carried out by either soaking in a solution of 10% Na_3PO_4 (for 15 to 30 min)[2,8] or dry sterilization (at 70°C for 1 to 3 days)[2,8]. However, our present data showed that although Na_3PO_4 or dry sterilization was able to reduce the abundance of PMMoV in infected seeds, the virus was still to some degree retained in the seeds. A similar result was also reported by Gao[2], who showed that soaking green pepper seeds in 10% Na_3PO_4 for 30 min could not disinfect tobamovirus completely. As reported earlier[12], in systemically infected plants, PMMoV was shown to propagate in the growing seeds, and the incidence of virus infection was almost 100% (see Fig. 1b). The procedure presented here is thus useful for disinfection of PMMoV in harvested seeds before cultivation.

Table 2 Efficient inactivation of PMMoV in infected seeds, assessed by their infectivity on *Nicotiana glutinosa*

Washed with[a]	Dry sterilization[b]	Numbers of necrotic local lesions developed on the leaves of *N. glutinosa*[c]
Distilled water	25°C	105.6±5.1***
Distilled water	70°C	29.3±9.1**
10% (w/v) Na_3PO_4	25°C	1.3±0.6*
10% (w/v) Na_3PO_4	70°C	0*

[a]Ten infected seeds were first washed with distillied water or 10% (w/v) Na_3PO_4 for 20 min.
[b]The seeds that had been washed with distillied water or 10% (w/v) Na_3PO_4 were further subjected to dry sterilization (70℃, 3h).
[c]Seeds were homogenized with 0.1 M phosphate buffer (0.1 ml per seed) and the homogenates were inoculated on the leaves of *N. glutinosa*. Numbers of necrotic local lesions developed on the inoculated leaves were measured 3 days after inoculation. The data show the means with standard devviations (S.D.) from three ewperiments. Asterisk numbers within the column indicate significant difference, as determined by the student's *t*-test ($P<0.01$).

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