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DEVELOPMENT OF A SIMPLE AND EFFECTIVE METHOD FOR SPECIFIC DETECTION OF PEPPER MILD MOTTLE VIRUS

Y. WANG[1], X. LI[2], Y. LIU[1], X. WANG[1*], G. ZHOU[1]

[1]State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, West Yuan Ming Yuan Road 2, Beijing 100193, P.R. China; [2]Institute of Plant and Environmental Protection, Beijing Academy of Agricultural and Forestry Sciences, Beijing, P.R. China

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Summary. – A digoxigenin (DIG)-labeled cDNA probe complementary to the region from 5,256–6,300 nt of Pepper mild mottle virus (PMMoV) genome was synthesized. The specificity and sensitivity of the probe was tested by the dot-blot hybridization. The detection limit of this method was equivalent to 0.8 μg of fresh infected tissue in each spot. Double-antibody sandwich (DAS) ELISA and RT-PCR had the detection limit 39 μg and 0.008 μg of fresh infected tissue, respectively. We evaluated leaf, fruit pulp, and seed of pepper plant by dot-blot hybridization and found all the tested tissues suitable for detection of PMMoV. Finally, 111 tissue samples including 93 samples collected from the pepper fields of Beijing and Baoding and 18 commercial seed samples were evaluated by this method. The results showed that the incidence rate of the infected samples was 14% and 61% for the field samples and commercial seeds, respectively. The high sensitivity and reliability of the molecular hybridization assay provided an important alternative method for the detection of PMMoV in a large-scale.

Keywords: Pepper mild mottle virus; digoxigenin-labeled cDNA probe; detection

Introduction

PMMoV is a species of the genus *Tobamovirus*. It can infect sweet and hot peppers (*Capsicum annuum* L.) expressing mosaic and malformations symptoms on the leaves and fruit that cause serious economic losses worldwide (Alonso *et al*., 1989; Nagai, 1981). The virus is transmitted mechanically or through seeds and contaminated soil (Tan *et al*., 1997; Lanter *et al*., 1982; Komuro and Iwaki, 1969).

In recent years, some newly introduced sweet pepper cultivars grown in greenhouses and fields of Beijing suburbs exhibited PMMoV-like disease symptoms. Afterward, the infectious agens was identified as PMMoV according to the biological assay and DAS-ELISA. The methods for control of this viral disease include quarantine systems to avoid introduction of foreign isolates, certification schemes to prevent virus spread, and cross-protection with mild isolates (Alonso *et al*., 1989; Nagai, 1981). All of these measures need rapid procedures to detect the virus. For this reason, a sensitive, specific, and reliable diagnostic method is needed.

ELISA and RT-PCR are two basic methods commonly used for the detection of PMMoV. RT-PCR is highly sensitive and specific, but it is time-consuming and prone to contamination. DAS-ELISA is used widely in a routine testing, but it is less sensitive than RT-PCR and needs a specific antibody (Du *et al*., 2007; Hossain *et al*., 2007; Kamenova and Adkins, 2004; Varveri *et al*., 2002; Hseu *et al*., 1987; Faris-Mukhayyish and Makkouk, 1983;

*Corresponding author. E-mail: xfwang@caas.net.cn; fax: +8610-62896114.

Abbreviations: DAS-ELISA = double-antibody sandwich ELISA; DIG = digoxigenin; PM = probe for PMMoV; PMMoV = Pepper mild mottle virus; PVY = Potato virus Y; TMV = Tobacco mosaic virus; ToMV = Tomato mosaic virus; SDS = sodium dodecyl sulfate; SSC = standard saline citrate

Koening, 1978). Non-radioactive molecular hybridization detection method is more sensitive than DAS-ELISA and can also efficiently process large numbers of samples (James, 1999; Sanchez-Navarro *et al*., 1996, 1998). Up till now, we have found no report about PMMoV detection using DIG-cDNA probe and no effort has been made to compare the sensitivity of the three techniques, RT-PCR, DAS-ELISA, and dot-blot hybridization using DIG-cDNA probe from the same PMMoV-infected sample.

The objective of this work was to develop an *in vitro*-transcribed DIG-cDNA probe for the detection of PMMoV by sensitive dot-blot hybridization method. We used this method as an alternative diagnostic tool for the testing of the field samples for large-scale survey.

Materials and Methods

Viruses and plants. The PMMoV-CN isolate was harvested from the diseased pepper plants in a Beijing greenhouse. After confirmation of the virus by DAS-ELISA, the complete sequence of the isolate was obtained and deposited in GenBank, Acc. No. AY859497. The PMMoV-CN isolate was maintained on sweet pepper plants (cv. Qiemen) in an insect-proof glasshouse. Cucumber mosaic virus (CMV), Tobacco mosaic virus (TMV), Tomato mosaic virus (ToMV), and Potato virus Y (PYV), which can also cause disease on pepper plant were also used in this study. During the season of 2006, a total of 111 pepper samples were collected including 18 brands of commercial seeds bought at seed markets and 93 pepper leaf samples showing PMMoV-like symptoms collected from the fields of Beijing and Baoding.

RNA extraction, RT-PCR, and amplification of viral RNA. Total RNA was extracted from 100 mg sample tissues by using Trizol Reagent (Invitrogen) following the manufacturer's instructions. After precipitation in ethanol, the total RNA was solubilized in 40 µl of nuclease-free water and reverse transcribed at 37°C for 60 mins by the cDNA synthesis system (Promega). Viral cDNA was amplified with upstream and downstream primers using AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen). Program of the thermal cycler was as follows: initial denaturation at 94°C for 2 mins, 25–35 cycles at 94°C for 30 secs; 55°C for 30 secs; extension at 68°C for 1 min. The reaction was carried out using a PCR system (Bio-Rad).

Molecular cloning and sequencing for cDNA fragment. Successful amplification of nucleotide segments of the expected size was confirmed by electrophoresis in 1% (w/v) agarose gel. The PCR products were purified using the DNA gel extraction kit (TaKaRa) and cloned into the pGEM-T vector (Promega). The recombinant vectors were transformed into *Escherichia coli* strain JM110. Plasmid DNA was obtained from colonies selected from overnight cultures by alkaline lysis. The cloned fragments were sequenced using the dideoxynucleotide chain termination method using an automated sequencer (Perkin-Elmer Applied Biosystems). At least three independently isolated clones were sequenced for each segment.

Table 1. PM primers and their sequences

Primer	Primer sequence (5'-3')	Binding site
PM-F	atttgccttcaaattgatcccg	5,256–5,277 bp
PM-R	tacatgtgtgacgtgtatttgcga	6,277–6,300 bp

Primer designing and generating specific probe. One pair of primers PM-F/PM-R was designed by Vector-NTI 7.1 software (Informax) for specific amplification of the sequence 5,256–6,300 nt in PMMoV genome to obtain the fragment of probe PM (Table 1) that was cloned and sequenced.

DIG-labeled cDNA probe was generated according to PCR DIG Probe Synthesis Kit (Roche Molecular Biochemicals). Species-specific probe for PMMoV was produced by PCR amplification of the cloned probe PM by the method performed as described in preceding part of this paragraph except of substitution of 4 nmol dNTPs with 1× PCR DIG labeling mix. The concentration of the probe was measured by using the Kodak Digital Science™ 1D image analysis software (Eastman Kodak).

Dot-blot hybridization assay. Total RNA was extracted from the sample tissue (100 mg) by using TRIzol reagent (Invitrogen). After precipitation with ethanol, total RNA was solubilized in 40 µl of nuclease-free water. Each RNA sample in the volume of 1 µl equivalent to 2.5 mg of fresh tissue was spotted onto nylon membranes Hybond-N+ (Amersham) soaked beforehand in 2× standard saline citrate (SSC). The nucleic acids were fixed to the membrane by drying at 120°C for 30 mins. The membranes could be pre-hybridized immediately or used later by storing them at 2–8°C.

Pre-hybridization and hybridization of dot-blots were performed following the manufacturer's instructions for nucleic acid blots with PCR DIG-labeled probes (Roche Applied Science). The hybridization solution contained 50 ng of probes for each ml of solution. Hybridization was carried out overnight at 50°C. The membranes were washed twice for 10 mins in 2× SSC and 0.1× sodium dodecyl sulfate (SDS) at room temperature and twice for 15 mins in 0.5× SSC and 0.1× SDS at 68°C. The hybridized probes were detected with anti-DIG-AP Fab fragments and stained for colorimetric detection with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT, Roche).

Sensitivity of dot-blot hybridization, DAS-ELISA, and RT-PCR. Pepper leaves collected from PMMoV-infected plants were cut into narrow strips and divided into 3 equal portions each of 100 mg and used in ELISA, dot-blot hybridization, and RT-PCR test, respectively.

For dot-blot hybridization and RT-PCR, the total RNA extracted from 100 mg of infected pepper leaves was solubilized in 40 µl and then serially diluted (5-fold dilution) in RNA dilution buffer (double-distilled water: 20× SSC:37% HCHO = 5:3:2). DAS-ELISA was essentially performed according to Clark and Adams (1977). 100 mg of infected or healthy pepper leaves were grinded in 1ml of buffer, extracted, and then serially diluted (2-fold dilution). The viral antigen was detected by PMMoV IgG and alkaline phosphatase-conjugated anti-IgG (Agdia Inc. Mishawaka Indiana). PMMoV-infected sample was considered as positive when A_{405} was at least two times higher than A_{405} of the non-infected control sample.

Results

Specificity analysis of the probe PM

One pair of primers (PM-F/PM-R) was designed to amplify 1,045 bp probe fragment from 5,256–6,300 nt in PMMoV genome. In order to evaluate the specificity of the primers to amplify the fragment of probe PM, the pepper plant tissues infected by ToMV, CMV, TMV, and PVY were used simultaneously in RT-PCR with PMMoV. The result showed that the 1,045 bp cDNA fragment was amplified from total RNAs from PMMoV-infected peppers by the primer pair PM-R/PM-F. No specific product was amplified by RNA prepared from ToMV-, CMV-, TMV-, and PVY-infected peppers, respectively (Fig. 1). The result showed that PM-R/PM-F was specific to the RNA isolated from PMMoV-infected plants.

The 1,045 bp cDNA fragment was cloned into the pGEM-T vector, sequenced, and identified as the expected fragment. The presence of DIG label in DNA made the labeled DNA to run slower in the gel than unlabeled DNA (data not shown).

In order to test the specificity of the probe PM in dot-blot hybridization, the nylon membrane was spotted with the RNA extracts from TMV-, CMV-, ToMV-, PVY-, and PMMoV-infected peppers. The DIG-labeled cDNA probe PM could react only with the total RNA extracted from PMMoV-infected leaves in dot-blot hybridization (Fig. 2). No hybridization signals were seen with total RNA extracted from the pepper leaves infected with other viruses and from healthy plants. The result showed that the PM probe was specific to the RNA extracted from PMMoV-infected plant.

Distribution of PMMoV in diseased pepper plant

To test the distribution of PMMoV in diseased pepper plant, total RNA from leaf, fruit pulp, and seeds of the same pepper plant were tested in dot-blot hybridization. All samples were spotted onto the same nylon membrane simultaneously. The total RNAs from the corresponding tissues of non-infected pepper plants were used as control. The result showed that all tested tissues including seed, leaf, and fruit pulp were identified as PMMoV-positive (Fig. 3). Thus, the leaf, fruit pulp, and seeds could be used as equivalent sources for RNA extraction and detection of PMMoV on pepper using the DIG-labeled cDNA probe.

Comparison of the sensitivity of dot-blot hybridization, DAS-ELISA, and RT-PCR

The sensitivity of the dot-blot hybridization was estimated by diluting the total RNA isolated from the PMMoV-infected leaf tissue. The probe could give a visible color reaction till the dilution 1:3,125 (5^{-5}), what corresponded to 0.8 µg of fresh weight tissue (Fig. 4). The dilution endpoint in RT-PCR was 1:3,125 (5^{-5}) what corresponded to 0.008 µg of fresh tissue (Fig. 5). In DAS-ELISA the reaction was positive up to the dilution 1:256 (2^{-8}), what corresponded to 39 µg of the fresh tissue (Fig. 6).

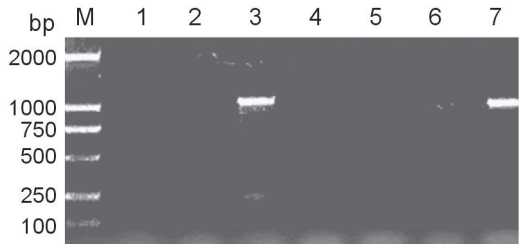


Fig. 1

Evaluating of the specificity of primers PM-R/PM-F by RT-PCR

TMV-infected (1), CMV-infected (2), PMMoV-infected (3), PVY-infected (4), ToMV-infected (5), and non-infected pepper plants (6); pGEM-T vector with the 1,045 bp fragment (7) and DNA size marker (M).

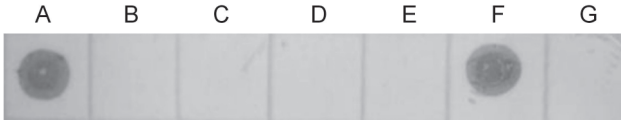


Fig. 2

Evaluating of the specificity of DIG-labeled cDNA probe PM by dot-blot hybridization

PMMoV-infected (A), TMV-infected (B), CMV-infected (C), ToMV-infected (D), PVY-infected (E), PMMoV-infected (F), and non-infected pepper plants (G).

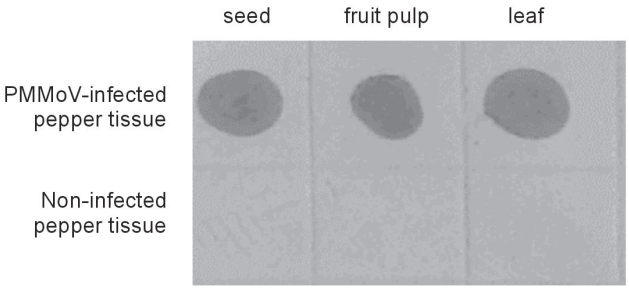


Fig. 3

Distribution of PMMoV in pepper plant detected by dot-blot hybridization

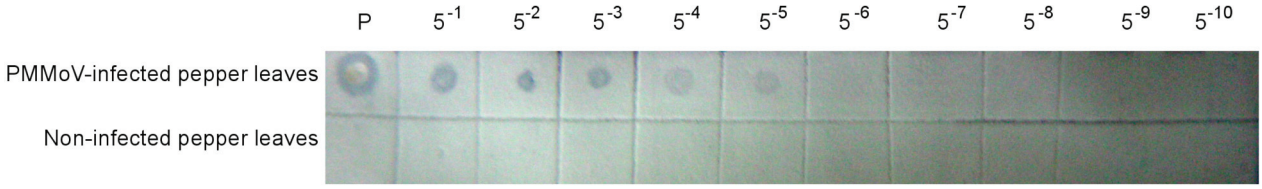


Fig. 4

Detection of different amounts of PMMoV RNA by dot-blot hybridization

Initial undiluted RNA (P) corresponds to total RNA extracted from 2.5 mg of the infected pepper leaves. Numbers at the top indicate the dilutions of PMMoV RNA.

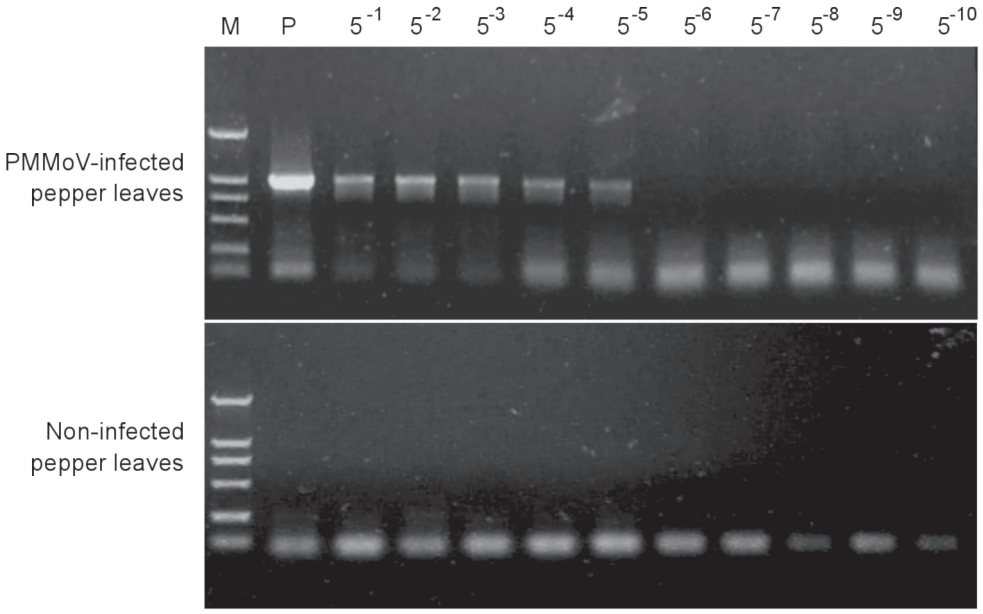


Fig. 5

Detection of different amounts of PMMoV RNA by RT-PCR

DNA size marker (M), initial undiluted RNA equivalent to 0.025 mg of infected pepper leaves (P). Numbers at top indicate the dilution of PMMoV RNA.

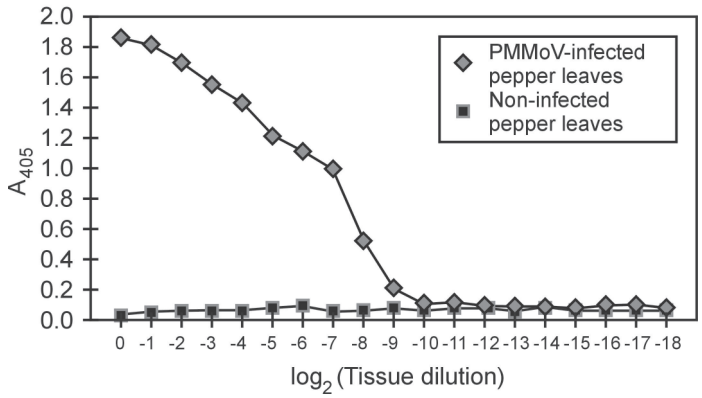
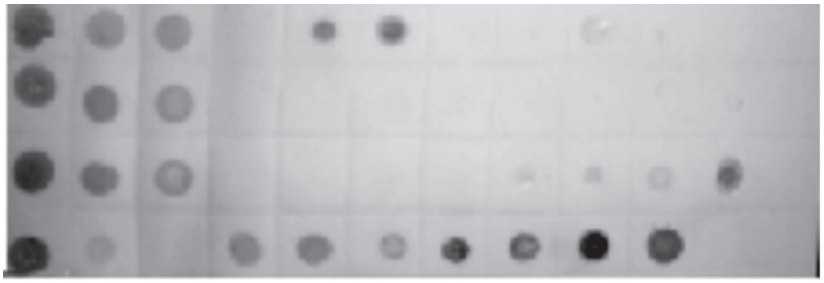


Fig. 6

Detection of different amounts of PMMoV antigen by DAS-ELISA

The antigen amount in the initial sample is equivalent to 10 mg of the infected leaves. Ordinate x: two-fold dilutions of extracts from infected and non-infected pepper leaves.



P	Z1	Z2	Z3	B4	S6	F1	M2	M11	M4	F5	H
Z4	Z5	Z6	Z7	B6	S5	F5	M7	M10	M1	F19	F11
Z8	Z9	Z10	Z11	B2	S11	F30	M8	M3	M5	F1	F14
Z12	Z13	Z14	Z15	B10	S13	F13	M9	M6	M13	F26	F2

Fig. 7

Dot-blot hybridization of 46 samples of pepper plants and seeds

Total RNA from PMMoV-infected (P) and healthy (H) pepper leaves. Seed samples (Z) and pepper plants collected from Baoding (B), Shunyi (S), Mentougou (M), and Fangshan (F).

The prevalence of PMMoV in pepper field samples and commercial seed samples

The DIG-labeled cDNA probe PM was used to detect PMMoV genomic RNA in 93 pepper leaf samples collected from 3 areas of Beijing and 1 area of Baoding and 18 commercial seed samples from seed markets in 2006. Total RNAs extracted from all samples were spotted onto the nylon membrane and hybridized with the probe PM. Out of 93 field pepper samples, 13 samples reacted positively with the PM probe including 2 of 37 samples (5.41%) collected from Shunyi, 7 of 13 samples (53.85%) from Mentougou, 2 of 31 samples (6.45%) from Fangshan, and 2 of 12 samples (16.67%) from Baoding (Fig. 7). Out of 18 seed samples, 13 samples reacted positively with the probe. The mean incidence rate of the infected field samples from the examined areas and the seed samples was 13.98% and 61.11%, respectively (Table 2). To determine the reliability of the method, all samples were tested also by the method of RT-PCR with the corresponding specific primers PM-R/PM-F. The numbers of positive samples were consistent with the results obtained by dot-blot hybridization assay (data not shown). The result demonstrated that the dot-blot hybridization assay was reliable in the routine detection of PMMoV.

Table 2. Detection of PMMoV in the leaf samples of peppers in Beijing and Baoding and commercial seeds by dot-blot hybridization

Collection areas	No. of samples	% of positives
Shunyi, Beijing	37	5.41
Mentougou, Beijing	13	53.85
Fangshan, Beijing	31	6.45
Baoding, Hebei	12	16.67
Total field samples	93	13.98
Seed samples	18	61.11

Discussion

PMMoV is an emerging tobamovirus found on pepper in northern China. Accurate identification and detection of the virus are the first steps in a successful management of the viral diseases. The present study described a non-radioactive dot-blot hybridization procedure to detect PMMoV. According to our knowledge this approach has not been applied by other researchers to detect the virus. Dot-blot hybridization using nucleic acid probes has been used by several research groups to detect and diagnose other plant viruses in the world, and its advantages were established (Hsu *et al*., 2005; Lee *et al*., 2001; Gioconda *et al*., 2000; James, 1999). The principal one is a more sensitive detection of viral RNA compared with serological methods and no need for virus-specific antibodies that are difficult to produce.

Because the suitable primer is a key factor for the specificity of the cDNA probe, the specific sequence present in the region from 5,256 to 6,300 bp of PMMoV genome was considered as conservative with a low similarity in comparison with the same regions of TMV, CMV, ToMV, and PVY, the other viruses usually infecting pepper in China. For that reason, one pair of primers was designed based on the sequence information currently available. This pair of primers could amplify specifically the corresponding se-

quence of PMMoV through RT-PCR and the DIG-labeled cDNA probe based on the pair of primers was specific for the detection of PMMoV.

The sensitivity of dot-blot hybridization, RT-PCR, and DAS-ELISA was tested in the same starting material. The detection sensitivity of these 3 procedures was equivalent to 0.8, 0.008, and 39 μg of fresh weight tissue, respectively. Dot-blot hybridization was more sensitive than ELISA, but less sensitive than RT-PCR in detection of PMMoV on pepper plants. The sensitivity of the dot-blot hybridization was similar to the results obtained by others. The dilution limit for detection of the leaves total RNA infected by TMV, CMV, and PVY was 1:1,000, 1:10,000 and 1:320, respectively, which was equivalent to 0.942 μg, 81.5 pg, and 1.8 μg of fresh leaf tissues (Du *et al*., 2004). Similarly, the sensitivity of this method for the detection of Citrus tristeza virus (CTV) could be as little as 0.1–1.0 mg of the infected tissues or 0.2 mg of fresh CTV-infected tissue (Barbarossa and Savino 2006; Gioconda *et al*., 2000). The detection limit of 3 DIG-labeled cDNA probes to detect the natural population of Barley yellow dwarf viruses in China were 25, 31.25, and 62.5 μg, respectively (Liu *et al*., 2007).

The dot-blot hybridization assay described by us was sensitive, reliable, economical, and provided a significant alternative to the serological methods that could be used specifically for the detection of PMMoV in a large scale.

By using the dot-blot hybridization method the incidence rate of PMMoV in the vicinity of Beijing in 2006 was evaluated. The detection results showed that the mean incidence rate of PMMoV in the vicinity of Beijing in 2006 was 13.98%, which was lower than the incidence of 71.4% found during 2003 and 2004 season (Wang *et al*., 2006). There are some reasons for this upturn. The first one may be better attention the farmer paid to the seed treatment and improvement in their farming conduction to prevent the virus introduction and spreading. The second reason represents environmental conditions in 2006 which were was less favorable for disease spreading. At last, the different material sampling also may cause the variation of incidence rate of the disease. The detection results also showed that the percentage of virus-containing commercial seeds was very high in our study. Like other tobamoviruses, PMMoV can be transmitted through seeds and can exist for a long time in the soil. Once a green pepper field has been contaminated with the virus, a de-contamination is very difficult and newly transplanted seedlings could be infected. So, surveillance of the spreading PMMoV throughout the China and seed-certifying need our immediate attention. The reliability of all results obtained by dot-blot hybridization were proved by the method of RT-PCR, which demonstrated that the dot-blot hybridization using DIG-labeled cDNA probe provided a reliable, rapid, and sensitive method to monitor spread of the disease in China.

In collaboration with several local laboratories, loads of samples could be collected and analyzed at the same time. Total RNA could be extracted from samples and spotted onto the nylon membrane straight at the collection site. Detection kits including DIG-labeled cDNA probes, nylon membranes, and the main reagents could be provided to the local laboratories of plant protection services. This approach has very significant implications for large-scale surveys as well as for the long-term epidemiological or ecological studies.

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