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Highly sensitive serological approaches for *Pepino mosaic virus* detection*

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Abstract: *Pepino mosaic virus* (PepMV) causes severe disease in tomato and other Solanaceous crops around globe. To effectively study and manage this viral disease, researchers need new, sensitive, and high-throughput approaches for viral detection. In this study, we purified PepMV particles from the infected *Nicotiana benthamiana* plants and used virions to immunize BALB/c mice to prepare hybridomas secreting anti-PepMV monoclonal antibodies (mAbs). A panel of highly specific and sensitive murine mAbs (15B2, 8H6, 23D11, 20D9, 3A6, and 8E3) could be produced through cell fusion, antibody selection, and cell cloning. Using the mAbs as the detection antibodies, we established double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), Dot-ELISA, and Tissue print-ELISA for detecting PepMV infection in tomato plants. Resulting data on sensitivity analysis assays showed that both DAS-ELISA and Dot-ELISA can efficiently monitor the virus in PepMV-infected tissue crude extracts when diluted at 1:1310720 and 1:20480 (weight/volume ratio (w/v), g/mL), respectively. Among the three methods developed, the Tissue print-ELISA was found to be the most practical detection technique. Survey results from field samples by the established serological approaches were verified by reverse transcription polymerase chain reaction (RT-PCR) and DNA sequencing, demonstrating all three serological methods are reliable and effective for monitoring PepMV. Anti-PepMV mAbs and the newly developed DAS-ELISA, Dot-ELISA, and Tissue print-ELISA can benefit PepMV detection and field epidemiological study, and management of this viral disease, which is already widespread in tomato plants in Yunnan Province of China.

Key words: *Pepino mosaic virus*; Monoclonal antibody; Serological method; Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA); Dot-ELISA; Tissue print-ELISA
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1 Introduction

Tomato (*Solanum lycopersicum*) is an important vegetable crop grown worldwide, and its yield and planting area are very huge, particularly in China and India. These plants suffer enormously from many viral diseases including *Pepino mosaic virus* (PepMV), which can devastate tomato production. PepMV was originally isolated from pepino in Peru in 1974, and

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has now become a widespread virus of greenhouse tomato plants worldwide, especially in Europe and North America (Jones et al., 1980; van der Vlugt et al., 2000; French et al., 2001; Pagán et al., 2006; Ling et al., 2008; Hanssen and Thomma, 2010). The hosts of PepMV were initially thought to be mainly Solanaceous species such as tomato, eggplant, potato, and species from the genera *Nicotiana*, *Datura*, *Capsicum*, and *Physalis* (Jones et al., 1980; Salomone and Roggero, 2002; Verhoeven et al., 2003). However, researchers have discovered that the virus can also be found in many other hosts including *Amaranthus* sp., *Calendula arvensis*, *Chrysanthemum segetum*, *Convolvulus arvensis*, and *Malva parviflora* (Jordá et al., 2001; Córdoba et al., 2004).

The PepMV-infected sweet pepino plants exhibit mosaic, dark green enations on the abaxial surfaces (Jones et al., 1980). Virus-affected tomato leaves show mosaic symptoms (Roggero et al., 2001). Other typical symptoms of PepMV-infected plants include yellow angular spots on the leaves, the so-called nettleheads, leaf scorching, leaf blistering or bubbling, and fruit marbling (Spence et al., 2006; Hanssen et al., 2008). PepMV is sap-transmissible and also readily spread mechanically by contaminated pruning and cultivating equipment and is, therefore, very contagious via regular crop handling procedures (Jones et al., 1980). In addition, bumblebees are suspected of spreading PepMV (Shipp et al., 2008) and seed transmission of PepMV has been reported in several studies albeit at relatively low levels (Córdoba-Sellés et al., 2007; Ling, 2008; Hanssen et al., 2010).

PepMV is a member in the genus *Potexvirus*, family Flexiviridae. PepMV virions are non-enveloped flexuous thread-shaped particles, 508 nm long and 13 nm wide (Jones et al., 1980). The viral positive single-stranded RNA genome is 6.4 kb in length and consists of five open reading frames (ORFs), 5' and 3' untranslated regions (UTRs), with a 3' poly-A tail. The viral genome encodes a 164-kDa RNA-dependent RNA polymerase (RdRp), three triple gene block (TGB) proteins of 26, 14, and 9 kDa (assigned TGBp1, TGBp2, and TGBp3, respectively), and a 25-kDa coat protein (CP) (Aguilar et al., 2002; Cotillon et al., 2002).

Currently, virus detection and roguing of infected plants are used to limit spread of PepMV infection, and constitute to the major approach for the prevention and control of the disease in the tomato industry (Hanssen and Thomma, 2010). Creating new, fast, simple, sensitive, inexpensive, and high-throughput detection approaches will greatly improve the ability of the industry to cost-effectively maintain healthy crops. Today, the methods described for detecting PepMV in plants mainly include reverse transcription polymerase chain reaction (RT-PCR) (Maroon-Lango et al., 2005; Pagán et al., 2006; Hasiów-Jaroszewska et al., 2009), immunocapture RT-PCR (IC-RT-PCR) (Mansilla et al., 2003), the RT-loop-mediated isothermal amplification (LAMP) assay (Hasiów-Jaroszewska and Borodynko, 2013), and polyclonal antibody (pAb)-based enzyme-linked immunosorbent assay (ELISA) and an immunochromatographic strip (Salomone and Roggero, 2002).

Although molecular techniques have been acknowledged to be specific and sensitive to detect PepMV, these techniques are not easily scalable. By contrast, serological assays are known to be quick, simple, low-cost, and high-throughput (Zhang MH et al., 2018; Zhang Y et al., 2020), although the effectiveness of serological methods depends primarily on the quality and availability of specific antibodies (Zhang et al., 2020). To develop sensitive serological techniques for PepMV detection, we prepared a panel of PepMV-specific monoclonal antibodies (mAbs) and further developed double antibody sandwich ELISA (DAS-ELISA), Dot-ELISA, and Tissue print-ELISA for highly sensitive and specific monitoring PepMV in plants. Since PepMV is already widespread in Yunnan Province, China, the mAbs and protocols developed in this study can be extremely useful and effective for PepMV detection, screening for PepMV-free seeds, and the management of this viral disease.

2 Materials and methods

2.1 Virus sources and plant samples

We generated an infectious clone of PepMV (Yunnan strain) in our laboratory (Li et al., 2018) and used it to inoculate *Nicotiana benthamiana* plants. Viral particles were then extracted from the infected plants following the methods described by Zhou et al. (1994). The prepared PepMV particles were used as the immunogen to generate murine hybridomas

secreting anti-PepMV mAbs. Twenty-three field tomato plant samples showing virus-like symptoms were gathered from tomato fields in Yunnan Province in China during 2019–2020. Known *Tomato yellow leaf curl virus* (TYLCV)-, *Tomato mottle mosaic virus* (ToMMV)-, *Tomato mosaic virus* (ToMV)-, *Tomato spotted wilt virus* (TSWV)-, or *Potato virus X* (PVX)-infected plants were stored in a −86 °C lab freezer.

2.2 Generation of murine mAbs

The prepared PepMV particles were used to immunize five eight-week-old BALB/c female mice through intraperitoneal injection (Song et al., 2017; Guo et al., 2020). Preparation of hybridomas and ascitic fluids containing mAbs was described previously (Wu et al., 2011; Huang et al., 2019). Titers of the mAbs were determined with an indirect-ELISA using the PepMV particles as the antigen. Isotypes of the mAbs were determined with a mouse mAb isotyping kit according to manufacturer's instructions (Sigma-Aldrich, MO, USA). Specificity and sensitivity of the produced mAbs were analyzed by antigen-coated-plate (ACP)-ELISA and western blot assays (Shang et al., 2011; Guo et al., 2020).

2.3 ACP-ELISA

We performed serial dilution tests to determine the optimal working dilutions of anti-PepMV mAbs and the alkaline phosphatase (AP)-conjugated goat anti-mouse IgG second antibody (Sigma-Aldrich) for ACP-ELISA as described previously (Shang et al., 2011; Song et al., 2017). Briefly, the plant tissues were ground and homogenized in a 0.05 mol/L sodium bicarbonate buffer. After centrifugation, the supernatants were coated to 96-well ELISA microplates (100 μL supernatant/well) and the plate was incubated overnight at 4 °C. After three rinses with 0.01 mol/L phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST, pH 7.4), all plate wells were blocked with 0.01 mol/L PBS containing 3% (0.03 g/mL) skimmed milk powder for 30 min. Each serially two-fold diluted anti-PepMV mAb solution (primary antibody) was added into each well of a vertical column of the plate and the plate was incubated at 37 °C for 1 h. After three rinses with PBST, each serially two-fold diluted AP-conjugated goat anti-mouse IgG second antibody was added into each well of a transverse row of the plate followed by 1 h incubation at 37 °C. The optical density at 405 nm (OD_{405}) of each well was measured with the microplate reader at 30 min post adding substrate. The supernatants from a PepMV-infected and a healthy tomato plant were used as the positive and negative controls, respectively. ACP-ELISA was performed as described by Zhang et al. (2020).

2.4 DAS-ELISA

Individual purified mAb IgG against PepMV was conjugated with AP (Sigma-Aldrich) as previously described (Chen et al., 2017). To develop DAS-ELISA for PepMV detection, the effectiveness of different combinations of capture mAb and AP-conjugated mAb IgG was analyzed. Serial dilution tests were also used to determine the working dilutions of the capture antibody and the AP-conjugated detection antibody for DAS-ELISA. DAS-ELISA procedure was carried out as described by Chen et al. (2017).

2.5 Dot-ELISA and Tissue print-ELISA

Dot-ELISA was performed as described previously with slight modifications (Wu et al., 2014; Zhang et al., 2020). Briefly, in a mortar, the collected plant tissues were ground and homogenized in 0.01 mol/L PBS at a ratio of 0.1 g tissue per 2 mL buffer. After being centrifuged at 5000*g* for 3 min, the supernatant was plant tissue crude extract. Approximately 2.0 μL of plant crude extract from each sample was spotted onto a nitrocellulose membrane. After being air-dried at room temperature (RT), the membrane was soaked for 30 min in blocking buffer followed by 1 h incubation in anti-PepMV mAb solution. After washing with PBST, the membrane was incubated for another 1 h in AP-conjugated goat anti-mouse IgG second antibody solution. After four washes with PBST, the membrane was immersed in nitro-blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3'-indolylphosphate (BCIP) substrate for color development. The samples that developed purple color within 20 min were considered PepMV-positive.

Tissue print-ELISA was carried out as the protocol described by Liu et al. (2017). Briefly, stems were obtained from PepMV-, TYLCV-, ToMMV-, ToMV-, TSWV-, or PVX-infected plants and healthy tomato plants. After being cut with a blade, the cut surface was quickly printed on a nitrocellulose

membrane for 3 s. The following procedure was the same as that of Dot-ELISA described above.

2.6 RT-PCR and sequence analysis

Based on *CP* gene sequence of PepMV in GenBank, the forward primer (5'-ATGGCTGACA ATACCCCAGT-3', nucleotide position 8122–8141) and the reverse primer (5'-AAGTTCTGGGGGTGCA TCAAT-3', nucleotide position 8812–8832) (PepMV-YN: MN939787) were designed to amplify the segment of PepMV *CP* gene in RT-PCR. RT-PCR detection of PepMV was carried out as described previously (Hasiów-Jaroszewska et al., 2009). The PCR-amplified gene segments were individually sequenced and aligned with PepMV *CP* genes in GenBank.

3 Results

3.1 Virus purification

PepMV particles were extracted from PepMV-infected *N. benthamiana* leaves by an ultracentrifugation method. The PepMV virion preparation was negatively stained with 1% phosphotungstic acid (pH 7.5), prior to electron microscopic observation. Under an electron microscope, we observed a large number of flexuous and thread-shaped virions with 508-nm length and 13-nm width in the virion preparation. Morphology of the virion is similar to the viruses in the genus *Potexvirus* (Fig. 1).

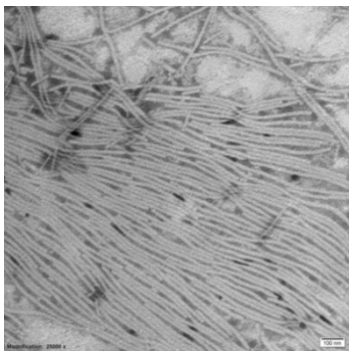

Fig. 1 Electron micrograph of the extracted PepMV virions
PepMV: *Pepino mosaic virus*. Bar=100 nm

3.2 Generation and characterization of anti-PepMV mAbs

On Day 3 after the third immunization, splenocytes were isolated from immunized mice and then fused with Sp2/0 myeloma cells to produce hybridomas. Via cell selection by hypoxanthine-aminopterin-thymidine (HAT) medium and anti-PepMV antibody detection by indirect-ELISA, we discovered 869 hybridoma wells containing anti-PepMV antibodies. Then, we selected 21 hybridomas secreting highly sensitive anti-PepMV antibodies for cell clone. Finally, six hybridoma lines (i.e., 15B2, 8H6, 23D11, 20D9, 3A6, and 8E3) secreting mAbs against PepMV were obtained by the limited dilution cloning. These hybridomas were then injected intraperitoneally into pristine-primed BALB/c mice for producing ascitic fluids containing mAbs. All selected mAbs were of the IgG1 isotypes with κ light chain (Table 1). Titers of all six mAbs in ascites were up to 1×10^{-7} through indirect-ELISA (Table 1). The IgG yields of mAbs from ascitic fluids ranged from 6.62 to 9.48 mg/mL (Table 1).

Western blot assay was further used to ascertain the specificity of the anti-PepMV mAbs. The resulting data showed that all six prepared mAbs could react consumingly and specifically with approximately 27-kDa protein in PepMV-infected tomato plant tissues or in the PepMV particle preparation (Fig. 2). We speculate that the detected protein band is PepMV CP based on the molecular weight of the reactive protein. No protein signals were observed in the lane loaded with the total protein extract from healthy tomato plant tissues (Fig. 2).

Serial dilution tests indicate that in ACP-ELISA, PepMV can be optimally monitored in the infected tomato plant tissues using 1:7000 (v/v) diluted mAbs (i.e., primary antibody) and 1:8000 (v/v) diluted AP-conjugated goat anti-mouse IgG second antibody. Specificity analysis experiment demonstrated that all six mAbs could specifically react with PepMV-infected tomato plant extracts, not with TYLCV-, ToMMV-, ToMV-, TSWV-, or PVX-infected or healthy plant tissue extracts (Fig. 3a), indicating that these six mAbs are specific for PepMV detection.

Sensitivity analysis using serially two-fold diluted crude extracts from a PepMV-infected or a healthy tomato plant tissue revealed that the mAbs (15B2, 8H6, 23D11, 20D9, 3A6, and 8E3) can monitor the virus in PepMV-infected tomato plant tissue crude extracts diluted at 1:163 840, 1:163 840, 1:81 920, 1:163 840, 1:81 920, and 1:81 920 (weight/volume ratio (w/v), g/mL), respectively (Fig. 3b), indicating that all six mAbs are highly sensitive for PepMV detection.

Table 1 Features of monoclonal antibodies (mAbs) to *Pepino mosaic virus* (PepMV)

mAb	Isotype and subclass	Ascites titer[1]	Yield of IgG in ascites (mg/mL)
15B2	IgG1, κ chain	1×10^{-7}	8.49
8H6	IgG1, κ chain	1×10^{-7}	7.76
23D11	IgG1, κ chain	1×10^{-7}	9.03
20D9	IgG1, κ chain	1×10^{-7}	7.54
3A6	IgG1, κ chain	1×10^{-7}	6.62
8E3	IgG1, κ chain	1×10^{-7}	9.48

[1]The ascites titer was ascertained by indirect-enzyme-linked immunosorbent assay (ELISA)

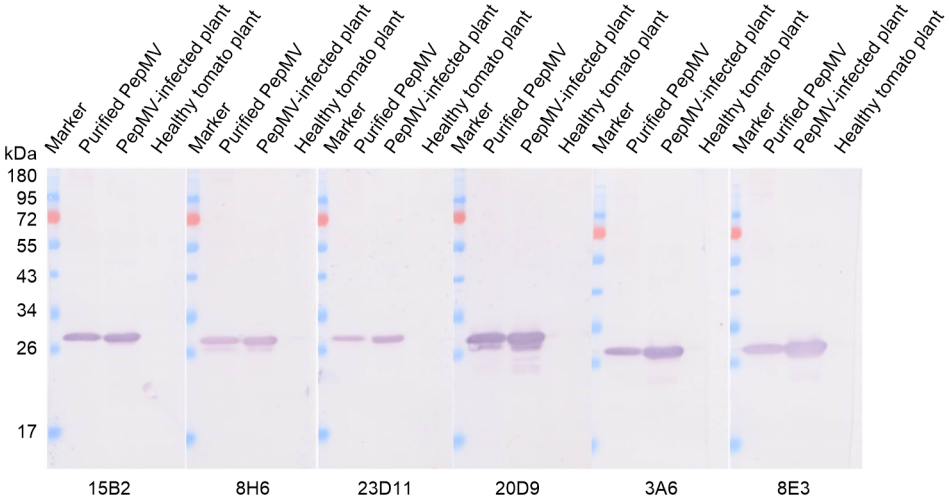

Fig. 2 Western blot assay of mAb specificity

Total plant protein was probed with prepared anti-*Pepino mosaic virus* (PepMV) monoclonal antibodies (mAbs). The identity of mAb is indicated at the bottom of the figure

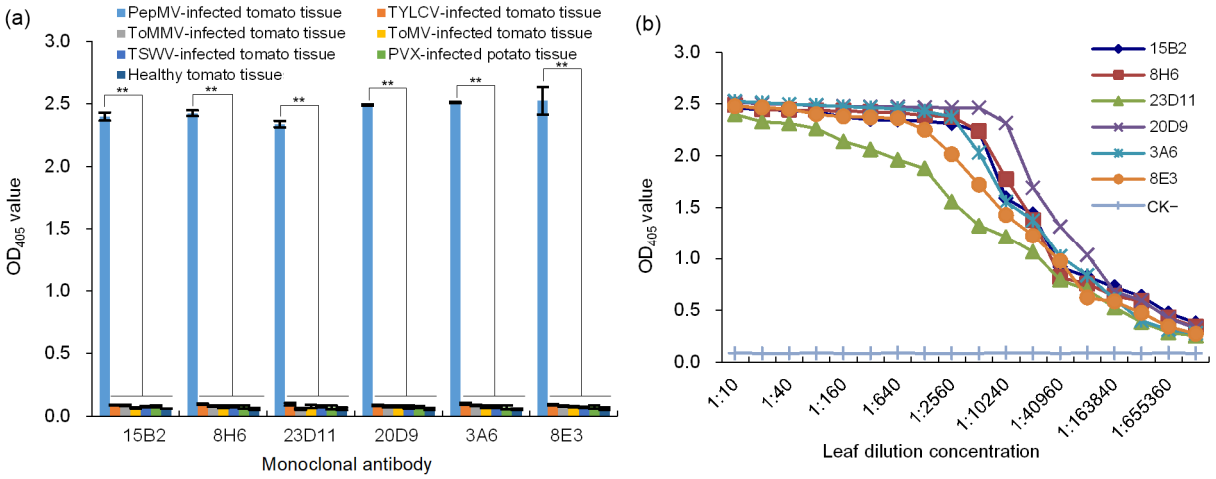

Fig. 3 Specificity and sensitivity analysis results of the prepared mAbs

(a) Crude extract from *Pepino mosaic virus* (PepMV)-infected tomato plants was diluted at 1:30 (weight/volume ratio (w/v), g/mL). (b) Two crude extracts from a PepMV-infected and a healthy tomato plant tissue were serially two-fold diluted from 1:10 to 1:2621440 (w/v) in the coating buffer, prior to use. The values of optical density at 405 nm (OD_{405}) were read at 30 min post adding the substrate at room temperature (RT) and expressed as mean±standard deviation (SD) of three biological replications. ** $P\leq0.01$. mAb: monoclonal antibody; CK−: a healthy tomato plant tissue used as a negative control

3.3 DAS-ELISA for PepMV detection

Our experiments showed that DAS-ELISA using mAb 8H6 as the capture antibody and AP-conjugated mAb 20D9 as the detection antibody gave the best detection effectiveness of PepMV. Serial dilution tests of DAS-ELISA showed that the optimal working dilutions for mAb 8H6 and AP-conjugated mAb 20D9 were 1:5000 and 1:8000, respectively. Serial two-fold dilutions of PepMV-infected or healthy tomato plant tissue crude extracts were used to determine the detection sensitivity of DAS-ELISA. Resulting data demonstrated that the detection endpoint of DAS-ELISA was 1:1310720 dilution for infected plant crude extracts (Fig. 4a). The specificity analysis result indicated that the DAS-ELISA could specifically detect PepMV infection in plants but had negative reactions for other viruses tested in this study (Fig. 4b). These data indicate that the developed DAS-ELISA is specific and sensitive to monitor PepMV infection in plants.

3.4 Dot-ELISA and Tissue print-ELISA for PepMV detection

The resulting data of serial dilution tests indicated that 1:6000 (v/v) diluted anti-PepMV mAbs (primary antibody) and 1:8000 (v/v) diluted AP-conjugated goat anti-mouse IgG secondary antibody were optimal working dilution for detecting PepMV in Dot-ELISA and Tissue print-ELISA. Specificity of the Dot-ELISA was confirmed using PepMV-infected tomato plant tissues and TYLCV-, ToMMV-, ToMV-, TSWV-, or PVX-infected or healthy plant tissues (Fig. 5a). Two crude extracts respectively from a PepMV-infected and a healthy tomato plant were serially two-fold diluted from 1:20 to 1:20480 (w/v, g/mL) and used to ascertain the detection endpoints of the developed Dot-ELISA. Sensitivity analysis experiment indicated that PepMV could be effectively monitored in 1:20480 (w/v, g/mL) diluted crude extracts from PepMV-infected tomato plant tissues (Fig. 5b).

To explore whether Tissue print-ELISA can be used to monitor PepMV infection in tomato plants, stems were transversely cut from known TYLCV-, ToMMV-, ToMV-, TSWV-, or PVX-infected, and healthy plants and the cut surfaces were printed individually on a nitrocellulose membrane. The printed membrane was probed by anti-PepMV mAb followed by the AP-conjugated goat anti-mouse IgG second antibody. After adding NBT/BCIP substrate, dark purple color indicating PepMV-positive was observed on the print made with the PepMV-infected tomato plant. No color development was found on the prints made with TYLCV-, ToMMV-, ToMV-,

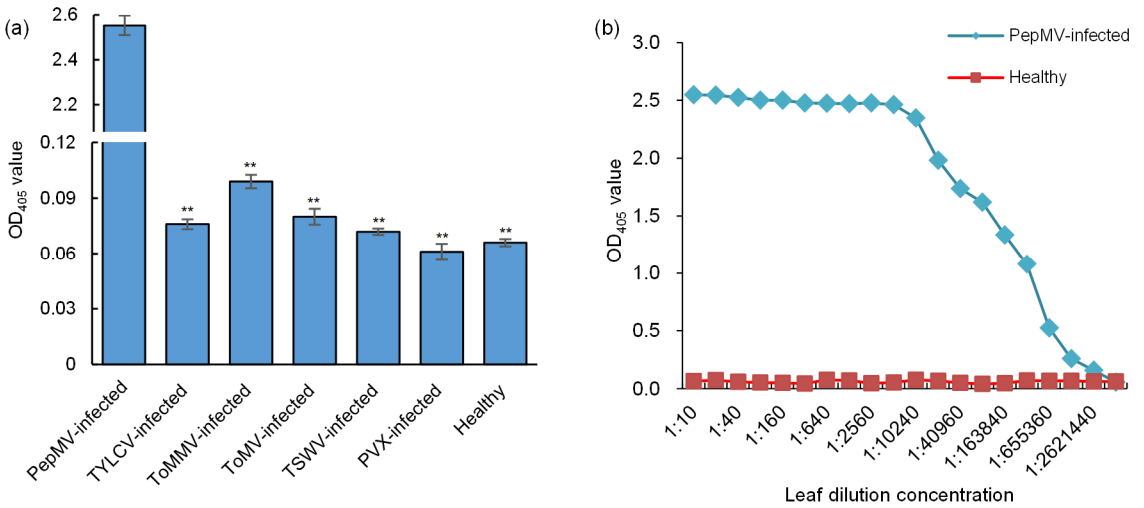

Fig. 4 Specificity and sensitivity analyses of the developed DAS-ELISA

(a) Six different viruses-infected extracts and a healthy tomato plant extract were diluted at 1:30 (weight/volume ratio (w/v), g/mL) in 0.01 mol/L phosphate-buffered saline (PBS) prior to use. The values of optical density at 405 nm (OD_{405}) were expressed as mean±standard deviation (SD) of three biological replications. ** $P \leq 0.01$ vs. *Pepino mosaic virus* (PepMV)-infected extract. (b) PepMV-infected extract and a healthy tomato crude extract were serially two-fold diluted in 0.01 mol/L PBS from 1:10 to 1:5242880 (w/v, g/mL). These diluted extracts were used to determine the sensitivity of the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). The OD_{405} values were read at 30 min post addition of the substrate at room temperature

Fig. 5 Specificity and sensitivity analysis results of the established Dot-ELISA

(a) Specificity analysis results of Dot-enzyme-linked immunosorbent assay (ELISA) for monitoring *Pepino mosaic virus* (PepMV) in tomato plants. On each membrane, two dots (upper and lower) were made using two crude extracts from PepMV-, *Tomato yellow leaf curl virus* (TYLCV)-, *Tomato mottle mosaic virus* (ToMMV)-, *Tomato mosaic virus* (ToMV)-, *Tomato spotted wilt virus* (TSWV)-, or *Potato virus X* (PVX)-infected plant tissues, or healthy tomato plant tissues. (b) Sensitivity analysis results of Dot-ELISA for monitoring PepMV in tomato plants. Two crude extracts from PepMV-infected and healthy tomato plant tissues were serially two-fold diluted from 1:20 to 1:20480 (weight/volume ratio (w/v), g/mL) in phosphate-buffered saline (PBS) (pH 7.4). Purple color represents a positive reaction. The mAb names were indicated on the right side of the figure

TSWV-, or PVX-infected, or healthy plant stems (Fig. 6), indicating the established Tissue print-ELISA was highly specific for PepMV and had no cross-reaction with the tested five other plant viruses.

3.5 Detection of PepMV infection in the field-collected tomato samples

The presence of PepMV in 23 field-collected tomato plants was screened simultaneously through the developed DAS-ELISA, Dot-ELISA, Tissue print-ELISA, and RT-PCR. Six of the twenty-three field samples tested positive for PepMV through these three serological approaches (Figs. 7a–7c). The results of the serological detection methods agreed with those obtained by RT-PCR method (Fig. 7d). Results of sequencing and sequence alignment of the resulting PCR products indicated that the PepMV isolates were detected in the field tomato plants and shared 82.0%–99.7% sequence similarity with PepMV *CP* genes in GenBank. Taken together, these data confirm that the newly developed DAS-ELISA, Dot-ELISA, and Tissue print-ELISA approaches were highly sensitive and accurate for detecting PepMV infection in plants. Furthermore, to our knowledge, this is the first report on PepMV infection in plants in China.

4 Discussion

Van der Vlugt et al. (2000) first reported that PepMV could infect tomato crops in the Netherlands. Now, PepMV has become an important virus on tomato plants worldwide, and causes serious economic losses because of its high rates of infection (French et al., 2001; Pagán et al., 2006; Spence et al., 2006; Ling et al., 2008; Hanssen and Thomma, 2010). Currently, strategies to prevent and control PepMV often fail because of limited understanding the disease (Hanssen and Thomma, 2010). Development of a simple, fast, sensitive, cost-effective, and high-throughput technique for detecting PepMV is crucial for studying its epidemiology and managing this disease.

Visual inspection is a common and simple method for diagnosing plant viral diseases in the fields. However, diagnosis based on visual inspection

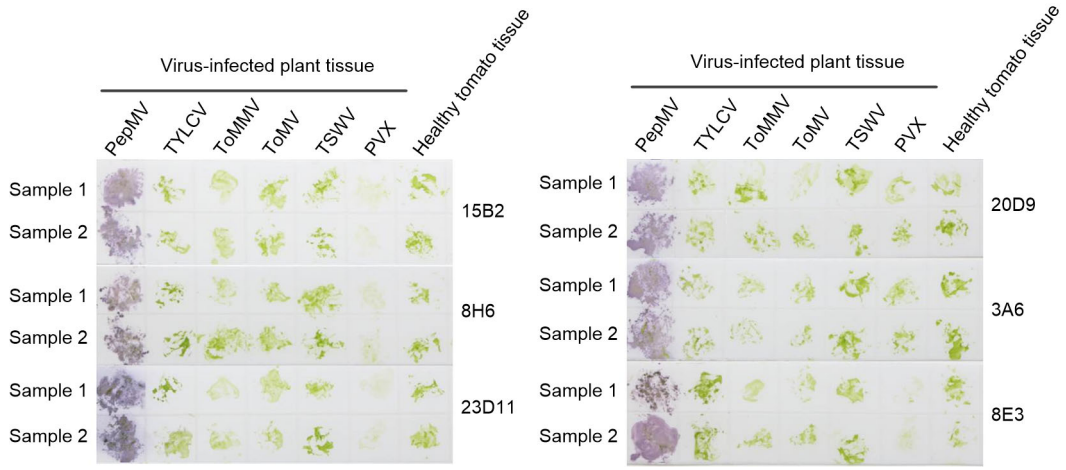

Fig. 6 Specificity analysis of the established Tissue print-ELISA

Specificity analyses of the established Tissue print enzyme-linked immunosorbent assay (ELISA). Columns from 1 to 6 were the prints from *Pepino mosaic virus* (PepMV)-, *Tomato yellow leaf curl virus* (TYLCV)-, *Tomato mottle mosaic virus* (ToMMV)-, *Tomato mosaic virus* (ToMV)-, *Tomato spotted wilt virus* (TSWV)-, and *Potato virus X* (PVX)-infected plant stems. A healthy tomato stem was used as the negative control. The prints were probed with anti-PepMV monoclonal antibodies (mAbs) followed by the alkaline phosphatase (AP)-conjugated goat anti-mouse IgG second antibody. Purple color prints indicated PepMV-positive, while light green color prints represented PepMV-negative

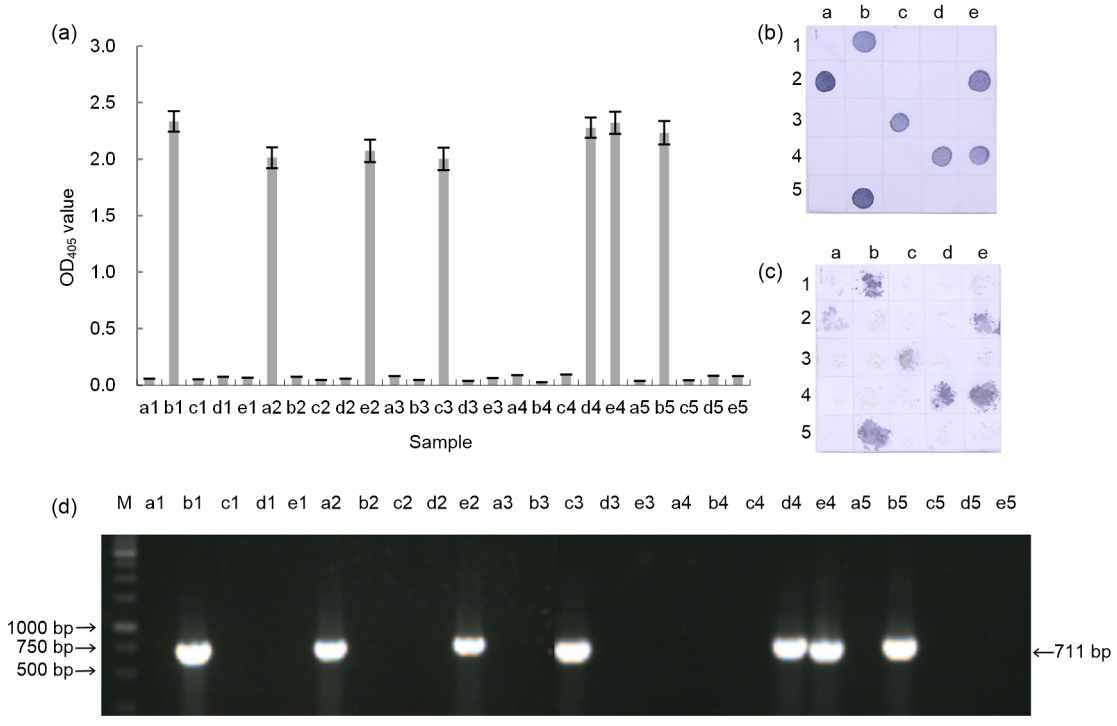

Fig. 7 Detection results of PepMV infection in field-collected tomato plants by DAS-ELISA (a), Dot-ELISA (b), Tissue print-ELISA (c), and RT-PCR (d)

Labels a1–a5, b1–b5, c1–c5, d1–d5, and e1–e5 represented individual field samples. Labels a1 and b1 were a known uninfected and a PepMV-infected tomato plant, and used as a negative and a positive control, respectively. Lane M is a 1 kb DNA ladder. PepMV: *Pepino mosaic virus*; DAS-ELISA: double antibody sandwich enzyme-linked immunosorbent assay; RT-PCR: reverse transcription polymerase chain reaction

is often inaccurate as symptoms of infection are often overlapping and PepMV infection can be easily confused with other pathogens or horticultural problems. The current diagnostic approaches for PepMV are primarily dependent on RT-PCR (Maroon-Lango et al., 2005; Pagán et al., 2006; Hasiów-Jaroszewska et al., 2009), IC-RT-PCR (Mansilla et al., 2003), and the real-time RT-PCR (Ling et al., 2007). RT-PCR is often used in laboratory for detecting plant RNA viruses because of its sensitivity and specificity. However, RT-PCR is costly, time-consuming, and not scalable. To overcome these constraints, the RT-LAMP assay was developed for PepMV detection (Hasiów-Jaroszewska and Borodynko, 2013). However, it is well known that the accuracy of RT-LAMP is not good enough because of a high false positive rate (Li et al., 2017). Compared with these PCR-based approaches above, serological methods are known to be rapid, simple, cost-effective, and suitable for high volume testing.

Serological techniques have been widely applied in detection and field surveys of plant viral diseases (Shang et al., 2011; Wu et al., 2011; Zhang et al., 2020). However, it is well known that the validity of a serological approach depends mainly on the sensitivity and specificity of the viral detection antibodies. Salomone and Roggero (2002) developed ELISA and an immunochromatographic strip for PepMV detection using the prepared pAbs with detection endpoints of both DAS-ELISA and the test strip for crude extracts from the infected plant tissues set at 1:8192 and 1:2048 dilution, respectively. However, these pAbs have been shown to have some drawbacks such as limited yield, high cross-reactions with other viruses in the same genus or with host proteins, leading to high background noise (Song et al., 2017). However, the serological approaches we developed in the current study are much more effective. mAb has these following merits: (1) researchers can use preliminary screening to select highly sensitive and specific mAbs; (2) once a hybridoma line that secretes mAb is available, unlimited number of mAb can be easily generated; (3) serological approaches based on the high-quality mAbs can obtain more reliable and accurate detection results than those using pAbs (Liu et al., 2014; Zhang et al., 2020).

The six produced mAbs in this study reacted strongly with PepMV in crude extracts from infected tomato plant tissues, but not with crude extracts from TYLCV-, ToMMV-, ToMV-, TSWV-, or PVX-infected plants, or from healthy tomato plants. To our knowledge, the six mAbs prepared in this study are the first ever reported about anti-PepMV mAbs use. Using the produced mAbs as the detection antibody, three serological approaches (i.e., DAS-ELISA, Dot-ELISA, and Tissue print-ELISA) were developed for specific and sensitive detection of PepMV infection in tomato plants. The dilution endpoints (i.e., sensitivities) of the developed DAS-ELISA and Dot-ELISA were 1:1310720 and 1:20480 (w/v, g/mL) dilution, respectively, for crude extracts from infected tomato plants. Surprisingly, the DAS-ELISA approach developed in this study is 160 times more sensitive than DAS-ELISA conducted by Salomone and Roggero (2002). We conclude that the Tissue print-ELISA is the fastest and most practical approach for PepMV test among these three newly established serological methods. The dependability of the three serological approaches for field plant sample detection was validated by RT-PCR followed by DNA sequencing. According to the field survey, we have demonstrated that PepMV is already widespread in Yunnan Province of China. In addition, tomato is one of the most important vegetable crops grown worldwide. Thus, we conclude that the three newly developed serological approaches are highly valuable for the diagnosis and management of this vial disease during tomato production all over the world.

PepMV causes a contagious disease in crops because of its simple transmission by contaminated contact, grafting, and other significant vectors. Shipp et al. (2008) proved that bumblebees contributed to the spread of PepMV since they are often used for pollination in commercial tomato production. In addition, and perhaps more seriously, several reports demonstrated that PepMV exists a low rate seed transmission, implying that there is a substantial risk associated with tomato seeds harvested from infected crops (Córdoba-Sellés et al., 2007; Ling, 2008; Hanssen et al., 2010). In addition, PepMV in dry plant materials is thought to remain viable for as long as three months, and the virus in moist organic debris is considered capable of infection for a relatively long period. In our next study, the virus carrying rate of bumblebees will be further investigated using the serological approaches developed here, and the feasibility

of the serological methods for detecting PepMV in tomato seeds will be analyzed.

In summary, the obtained six anti-PepMV mAbs and the three newly established serological approaches are very useful for PepMV detection and epidemiological study, and establishment of prevention and control strategies for this viral disease. Furthermore, to our knowledge, this is the first report on PepMV infection in plants in China.

Contributors

Wan-qin HE and Yi-yi REN prepared the mAbs and drafted the manuscript. Jia-yu WU conducted Dot-ELISA and Tissue print-ELISA experiments. Ya-juan QIAN and Song-bai ZHANG collected the samples. Xue-ping ZHOU, Fang-fang LI, and Jian-xiang WU conceived the study and revised the manuscript. Jian-xiang WU proof-read and finalized the manuscript. All authors have read and approved the final manuscript and, therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Wan-qin HE, Jia-yu WU, Yi-yi REN, Xue-ping ZHOU, Song-bai ZHANG, Ya-juan QIAN, Fang-fang LI, and Jian-xiang WU declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：凤果花叶病毒高灵敏度血清学检测技术

目　的：建立基于单克隆抗体的检测番茄等植物中凤果花叶病毒（PepMV）的血清学方法，为 PepMV 的田间调查和诊断及其科学防控提供快速实用的检测技术。

创新点：首次制备了抗 PepMV 的高度特异和灵敏的单克隆抗体，并利用制备的单抗建立了 3 种能特异且灵敏地检测 PepMV 的血清学方法。

方　法：以差速离心方法提纯的 PepMV 粒子作为免疫原免疫 BALB/c 小鼠，通过杂交瘤技术获得了能稳定传代并分泌 PepMV 单克隆抗体的杂交瘤细胞株；杂交瘤细胞注射到小鼠腹腔获得单克隆抗体腹水，并以制备单抗为核心，根据血清学原理建立检测植物中 PepMV 的双抗夹心酶联免疫吸附试验（DAS-ELISA）、斑点酶联免疫吸附试验

（Dot-ELISA）和组织印迹酶联免疫吸附试验（Tissue print-ELISA）三种血清学检测方法；利用田间番茄样品分析建立的血清学方法检测PepMV的有效性。

结　论：利用杂交瘤技术获得了6株能分泌高度特异灵敏PepMV单克隆抗体的杂交瘤细胞株，以分泌的单抗为核心建立了检测植株中 PepMV 的 DAS-ELISA、Dot-ELISA 和 Tissue print-ELISA 三种高度灵敏的血清学新技术。三种建立的血清学技术检测感染 PepMV 的番茄植株均呈强阳性反应，而检测健康番茄及感染其他5种植物病毒的植株呈阴性反应，且 DAS-ELISA 和 Dot-ELISA 血清学技术检测番茄病叶粗提液的灵敏度分别达到1:1310720 和 1:20480 倍稀释（质量体积比，g/mL）。田间样品检测结果发现，建立的血清学技术的检测结果与反转录聚合酶链反应（RT-PCR）的检测结果一致，表明建立的血清学方法可有效地用于植物中 PepMV 的检测。同时，本研究首次发现 PepMV 已在我国云南番茄作物上发生流行。PepMV单克隆抗体的制备及其灵敏血清学检测方法的建立有益于 PepMV 的田间调查和诊断及其科学防控。

关键词：风果花叶病毒；单克隆抗体；血清学方法；双抗夹心酶联免疫吸附试验（DAS-ELISA）；斑点酶联免疫吸附试验（Dot-ELISA）；组织印迹酶联免疫吸附试验（Tissue print-ELISA）