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Development of polyclonal antisera against movement proteins from three poleroviruses infecting cucurbits

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Abstract

Cucurbit aphid-borne yellows virus (CABYV), melon aphid-borne yellows virus (MABYV) and suakwa aphid-borne yellows virus (SABYV) are three poleroviruses that infect cucurbit crops. Developing specific antisera against such viruses is crucial for their detection and functional understanding of related genes. However, no studies have yet reported viral detection using antisera against movement proteins (MP) in these three viruses. In this study, we generated plasmids expressing three viral MP genes, and transformed them into the *Escherichia coli* strain, *Rosetta*, to recombinantly express and purify fusion proteins. Then, polyclonal antisera were derived by immunizing New Zealand white rabbits, after which western blotting was used to determine the titer, sensitivity and specificity of the antisera. The antisera titers against MP^{CABYV}, MP^{MABYV} and MP^{SABYV} were 1:512000, 1:256000 and 1:256000, respectively. The optimized working concentrations for the three antisera ranged between 1:10000 and 1:64000. Additionally, antisera against MP^{CABYV} and MP^{MABYV} only reacted with their corresponding MP proteins. Antiserum against MP^{SABYV} not only had the strongest reaction with its MP, but also reacted weakly with MP^{CABYV} and MP^{MABYV}. All three antisera exerted no serological reactions with other poleroviruses. Furthermore, our data showed that all antisera specifically detected MPs in both *Nicotiana benthamiana* and cucumber leaves. Thus, we have established a system that sensitively detects three poleroviruses infecting cucurbits, using antisera against MPs. We provide a foundation for future research on the serological detection of these viruses, and interaction mechanisms between viruses and host plants.

Keywords: Cucurbit aphid-borne yellows virus, Melon aphid-borne yellows virus, Suakwa aphid-borne yellows virus, Movement protein, Prokaryotic expression, Antiserum preparation, Virus detection

Background

Cucurbits are important fruit crops across the world. Plant viruses that infect cucurbits cause significant economic losses and limit cucurbits production (Gholamali-zadeh et al. 2008; Knierim et al. 2014; Chikh-Ali et al. 2019). Cucurbit aphid-borne yellows virus (CABYV),

melon aphid-borne yellows virus (MABYV) and suakwa aphid-borne yellows virus (SABYV) are three important viruses infecting cucurbits, which belong to the *Polerovirus* genus, part of the *Luteoviridae* family (Lecoq et al. 1992; Xiang et al. 2008a; Shang et al. 2009). CABYV was first reported in France in 1992, where it infected cucurbit crops, and was later found in several other countries, including China (Lecoq et al. 1992; Lemaire et al. 1993; Abou-Jawdah et al. 1997; Juarez et al. 2004; Mnari Hat-tab et al. 2005; Tomassoli and Meneghini 2007; Xiang et al. 2008b; Bananej et al. 2009; Orfanidou et al. 2014). Interestingly, CABYV also naturally infects passion fruit and pepper, causing large yellow areas on leaves (Vidal

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et al. 2018; Liu et al. 2019; Zhang et al. 2019). MABYV and SABYV were first reported in China in 2008 and 2009, respectively (Xiang et al. 2008a; Shang et al. 2009). MABYV is widely distributed across several provinces in China and Thailand, however SABYV has only been detected in coastal provinces, such as Fujian and Guangdong, in southern China (Shang et al. 2009; Knierim et al. 2014). SABYV was also reported in several Southeast Asian countries, such as Thailand, the Philippines and East Timor (Knierim et al. 2010; Maina et al. 2016; Cheewachaiwit et al. 2017). All three viruses are limited to the phloem tissue of host plants and are transmitted by aphids in a persistent circulative and non-propagative manner (Hogenhout et al. 2008; Brault et al. 2010). These three viruses are highly specific to particular vectors and cannot be transmitted mechanically (Brault et al. 2010). The main symptoms of these viral diseases include yellowing and thickening of old leaves, which result in severe reductions in crop yield, however, all three viruses exert no significant effects on fruit quality and shape (Lecoq et al. 1992; Lemaire et al. 1993; Xiang et al. 2008a; Shang et al. 2009).

The poleroviruses virion, including CABYV, MABYV and SABYV, is a ball-shaped icosahedron with a diameter of 25–30 nm, encapsulating the genome RNA. The virions are relatively stable and not sensitive to chloroform and non-ionic detergents, but are destroyed under high-salt conditions (D'Arcy and Domier 2005). The genome of all three viruses consists of a single positive-RNA strand, approximately 5674–5843 nucleotides in length. It contains seven open reading frames (ORFs) (Additional file 1: Figure S1). The first three ORFs are expressed from genomic RNA and the others are expressed from subgenomic RNA (Guilley et al. 1994; Smirnova et al. 2015). Specifically, the P0 protein encoded by ORF0 is a typical RNA silencing suppressor, which enhances viral pathogenicity, and promotes viral accumulation by interacting with host plant genes (Prufer et al. 2006; Han et al. 2010; Zhuo et al. 2014; Sun et al. 2018; Li et al. 2019; Rashid et al. 2019). The P1 protein encoded by ORF1 serves as a protease, helicase and viral genome-linked protein (VPg). A P1-P2 fusion protein encoded via a ribosomal frameshift event has replicase activity which regulates poleroviruses replication in hosts, and serves as a marker for viral detection and identification (D'Arcy and Domier 2005; Yahaya et al. 2019). The intergenic-non-coding region (intergenic-NCR) is approximately 80 nt in length, and is located between ORF2 and ORF3a. The P3a protein which substitutes an AUG initiation codon with ACG is encoded by ORF3a and is indispensable for viral long-distance movement. When the conserved proline 18 in P3a has the non-synonymous substitution, viruses lose their systemic infection (Smirnova et al. 2015; Zhang et al. 2018). The coat protein (CP) is encoded by ORF3,

which participates in virion assembly, long-distance movement, and aphid-mediated poleroviruses transmission (Ziegler-Graff et al. 1996; Lee et al. 2005; Hipper et al. 2014). The read-through protein, encoded by ORFs 3 and 5, is closely related to aphid transmission, virion assembly, long-distance movement, phloem limited viral infection, and its α -helix formed in the C-terminus is essential to systemic infection and symptom expression (Brault et al. 1995; Bruyère et al. 1997; Peter et al. 2009; Hipper et al. 2014; Xu et al. 2018). MP encoded by ORF4 was located in the plasmodesmata, mitochondria, and chloroplast, and is associated with viral replication, cell-to-cell movement and long-distance movement (Sokolova et al. 1997; Lee et al. 2002; Hipper et al. 2013; Chen et al. 2018; DeBlasio et al. 2018). However, the function of MP on long-distance movement remains unclear.

Currently, poleroviruses are detected mainly by using reverse transcription polymerase chain reaction (RT-PCR) (He et al. 2006; Knierim et al. 2010). Multiple RT-PCR was ever used to detect CABYV, MABYV, SABYV, and identify three distinct brassica yellow virus (BrYV) genotypes (Shang et al. 2012; Zhang et al. 2015). In terms of serological investigation of these three viruses, only antiserum against CABYV, prepared by purified virions, was used for western blotting or enzyme-linked immunosorbent assay (ELISA) (Lecoq et al. 1992), however, it is hard to purify virus particles due to its low accumulation in the host, and the virus detection result by the anti-CP antiserum may not prove whether the virus is in active or not. In contrast, the positive results of MP detection indicated that such viruses may be replicating in hosts (Yang et al. 2019). Recent studies have shown that some viruses from the *Luteoviridae* family, including barley yellow dwarf virus PAV (BYDV-PAV) and potato leafroll virus (PLRV) can be detected by corresponding polyclonal antiserum against MPs (Chay et al. 1996; Yang et al. 2019; Hu et al. 2020). However, no studies have yet developed polyclonal antiserum against MPs of these three cucurbit-infecting viruses, or investigated their serological inter-relationships. In our work, to generate polyclonal antisera, His-MBP-MP purified fusion proteins, recombinantly produced in prokaryotic expression systems, were used to immunize New Zealand rabbits. Western blotting analysis showed that all viral MPs were specifically detected by their own antisera. We further confirmed serological relationships among them, and provide fundamental tools for future research on the serological detection of these viruses, and interactions between viruses and host plants.

Results

Prokaryotic MP expression, purification and production of antisera

MP^{CABYV}, MP^{MABYV} and MP^{SABYV} genes were amplified by PCR and cloned into the prokaryotic expression

vector pDB-His-MBP, respectively. After that, the three recombinant vectors were individually transformed into the *E. coli* strain, *Rosetta*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that a 65-kDa band corresponding to His+MBP + MP was generated by all three recombinant proteins, indicating successful protein expression and purification. His-MBP-CABYV-MP fusion protein was eluted with 200 mM and 500 mM imidazole (Fig. 1a). His-MBP-MABYV-MP fusion protein was eluted with 100 mM and 200 mM imidazole (Fig. 1b), and His-MBP-SABYV-MP fusion protein was eluted with 80–200 mM imidazole (Fig. 1c). Three mg of each concentrated protein was then subcutaneously injected into New Zealand white rabbits, and approximately 30 mL of each polyclonal antiserum was generated.

Antisera titer analysis

To determine the titer of three antisera we obtained, MP^{CABYV}, MP^{MABYV}, and MP^{SABYV} genes were cloned into the transient expression vector, pMDC32, and then were individually delivered into *N. benthamiana* leaves

via agro-infiltration method. Injection of the pMDC32 empty vector was used as a negative control. All three MPs were transiently expressed in *N. benthamiana* plants and their leaves were collected at 3 days post inoculation (dpi) with *Agrobacterium* suspension to extract total proteins. Western blotting results showed that all antisera detected their corresponding MPs. Furthermore, antiserum against MP^{CABYV} detected MP^{CABYV} protein even when it was diluted to 1:512000 (Fig. 2a). However, antisera against MP^{MABYV} and MP^{SABYV} detected their corresponding proteins at 1:256000 dilution (Fig. 2b, c). From the perspective of color appearance and economics, the optimal working concentration of the three antisera ranged from 1:10000 to 1:64000.

Antisera sensitivity analysis

To analyze the sensitivity of three antisera, total proteins from *N. benthamiana* leaves expressing MP^{CABYV}, MP^{MABYV}, and MP^{SABYV} were extracted respectively and serially diluted in equal proportions. Western blotting results showed that when antisera against MP^{CABYV}, MP^{MABYV}, and MP^{SABYV} were diluted to 1:1000, they

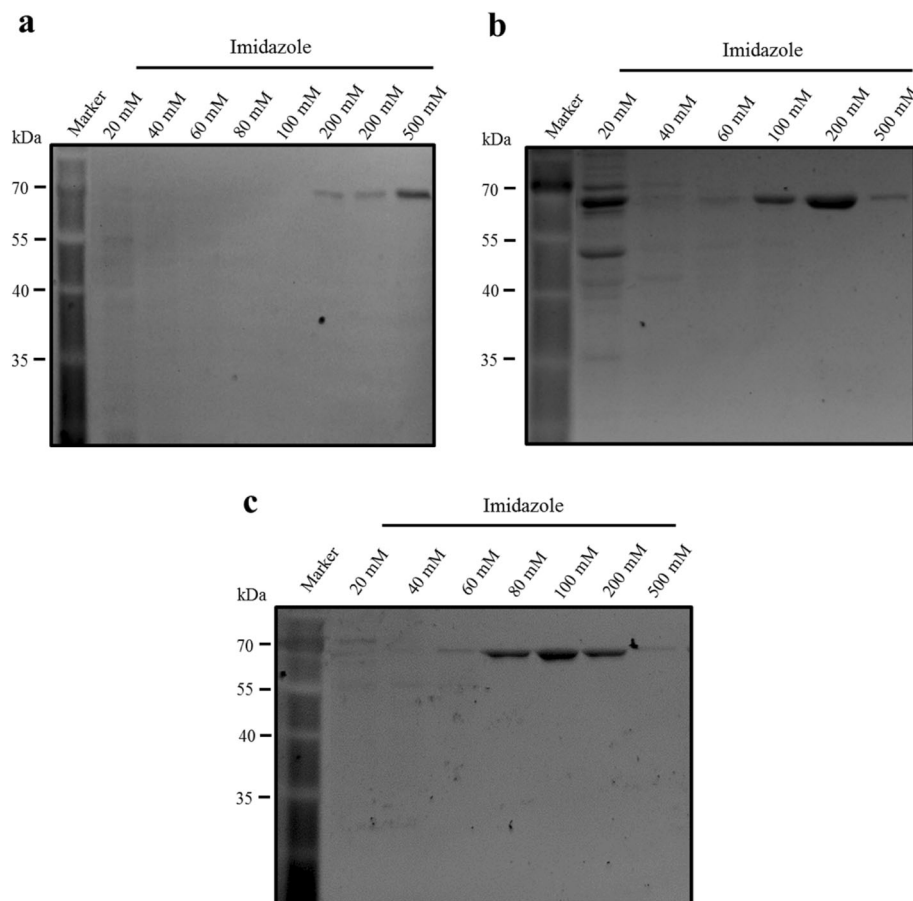
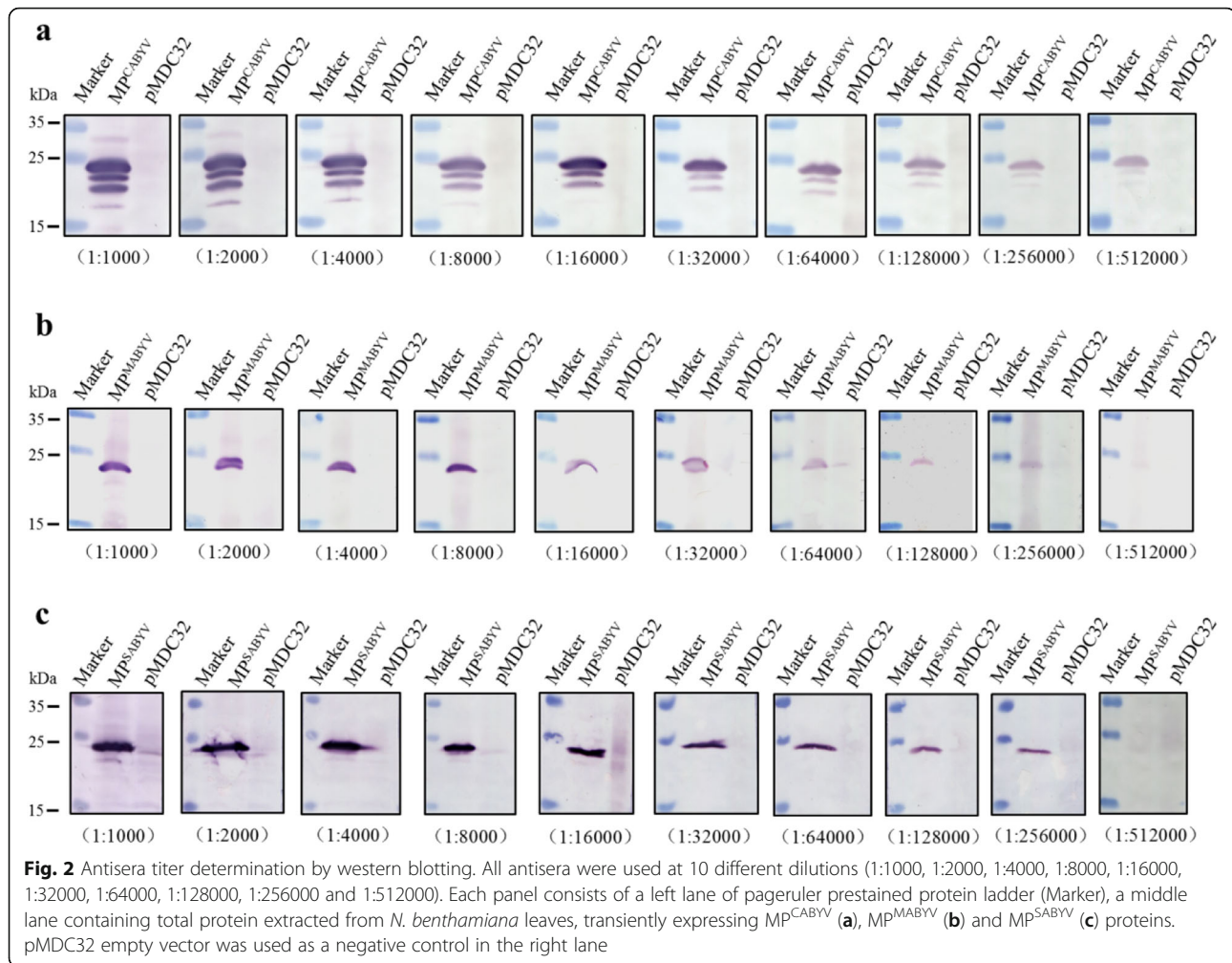


Fig. 1 SDS-PAGE analysis of CABYV-MP (a), MABYV-MP (b) and SABYV-MP (c) recombinant proteins. The first lane shows pageruler pre stained protein ladder (Marker), and the other lanes are fusion proteins eluted with seven different concentrations of imidazole



detected their corresponding protein samples diluted 128, 64, and 40 times, respectively. Subsequently, when these three antisera were diluted to 1:10000, they detected their protein samples diluted to 80, 16, and 16 times, respectively. When we diluted antisera to 1:20000, total protein samples corresponding to MP^{CABYV} diluted to 32 times were still detectable, so antiserum against MP^{CABYV} showed the highest sensitivity. In contrast, under the same conditions, antisera against MP^{MABYV} and MP^{SABYV} exhibited a lower sensitivity to their corresponding protein samples, and samples diluted to 8 times were detected (Fig. 3).

Antisera specificity analysis

Strong serological cross-reactions exist between different poleroviruses (D'Arcy and Domier 2005), therefore, it was necessary to determine whether antisera specifically detected target viruses. To analyze the specificity of three antisera, several MPs including MP^{CABYV}, MP^{MABYV}, MP^{SABYV}, 3Flag-MP^{BrYV}, 3Flag-MP^{PLRV} and 3Flag-MP^{ScYLV} (sugarcane yellow leaf virus) constructed in

pMDC vector were transiently expressed in *N. benthamiana* leaves via agro-infiltration. Injection of the pMDC32 empty vector was used as a negative control. Inoculated *N. benthamiana* leaves were collected at 3 dpi with *Agrobacterium* suspension to extract total proteins for western blotting analysis. All antisera were used at three dilutions (1:1000, 1:10000 and 1:20000). Antisera against MP^{CABYV} and MP^{MABYV} only consistently detected their corresponding MPs in total protein samples, regardless of the dilution level of antisera, indicating that these two antisera had a high specificity towards their target proteins (Fig. 4a, b). In the case of antiserum against MP^{SABYV}, it had the strongest reaction with MP^{SABYV} at all three dilution levels, and it also reacted with MP^{CABYV} and MP^{MABYV} at a weaker level. This meant that antiserum against MP^{SABYV} could specifically detect three different poleroviruses infecting cucurbits (Fig. 4c). Furthermore, all three antisera had no serological cross-reactivity with other MPs from the same genus, including MP^{PLRV}, MP^{BrYV} and MP^{ScYLV}.

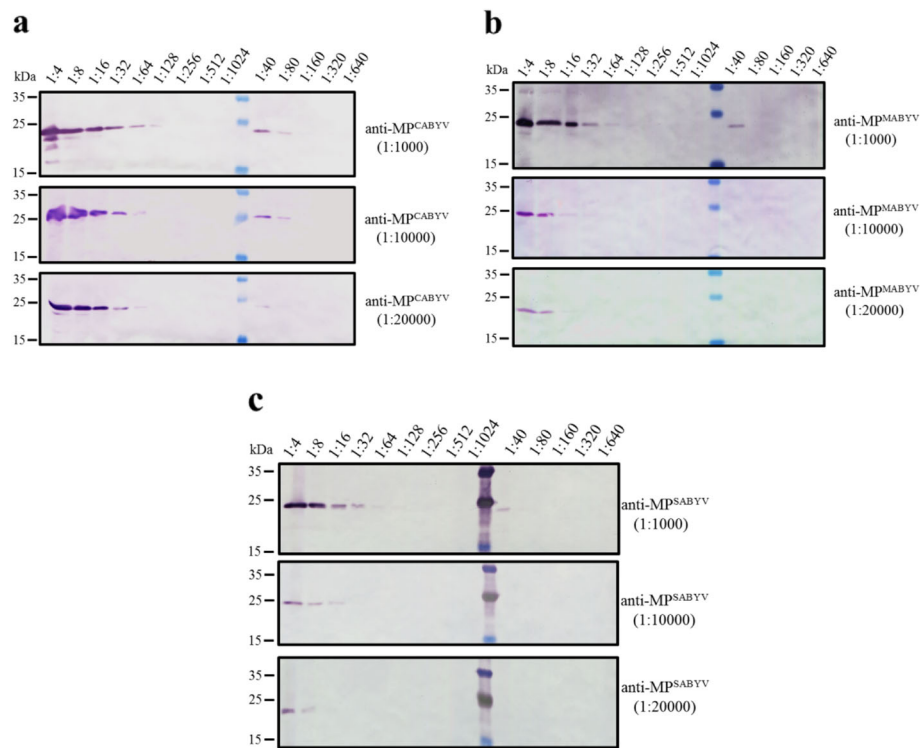


Fig. 3 Sensitivity analysis of antisera by western blotting. Marker: pageruler prestained protein ladder. The remaining lanes show several different dilutions of protein samples extracted from *N. benthamiana* leaves (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:40, 1:80, 1:160, 1:320, 1:640). Anti-MP^{CABYV} (a), anti-MP^{MABYV} (b) and anti-MP^{SABYV} (c) were used at 1:1000, 1:10000 and 1:20000, respectively

Application of antisera to viral detection

CABYV and MABYV full-length infectious clones were inoculated into *N. benthamiana* leaves, via agro-infiltration (Prüfer et al. 1995; Xiang 2011). Inoculated leaves were harvested at 3 dpi to extract total proteins for further western blotting analysis. Our data showed that antisera specifically detected CABYV and MABYV in *N. benthamiana* leaves, which agreed with RT-PCR detection results (Fig. 5a, b), indicating that our antisera were suitable for detecting viruses in a model plant, *N. benthamiana*.

Simulation detections were conducted to test whether antisera detected MPs in natural hosts of these viruses, such as cucumbers, due to a lack of available natural cucurbits samples infected by these three viruses. Diluted protein-containing transiently expressed MPs in *N. benthamiana* leaves were mixed with healthy cucumber leaf extracts. Western blotting results showed that protein samples diluted 256, 64 and 128 times were specifically detected by antisera against MP^{CABYV}, MP^{MABYV} and MP^{SABYV}, respectively (Fig. 5c). Total proteins from healthy cucumber leaves exerted no serological reactions with these three antisera, and no interference was observed with specific serological responses of the three antisera to their corresponding MP proteins.

Discussion

Virus infection depends on its efficient movement in hosts. Blocking virus movement provides strategies for host plants to defend against harmful viruses, therefore it is important to study how MPs participate in virus movement in hosts. MPs play essential roles in virus infection. They not only promote intercellular movement and systemic viral movement but also serve as critical pathogenic factors, causing purple symptoms in infected leaves (Chen et al. 2018). Thus, we established a specific viral detection system by developing antisera against three different MPs. Such efforts are indispensable for studying viral occurrence and distribution, potentially revealing MP functions at the protein level.

Antisera against MP^{CABYV} and MP^{MABYV} showed higher protein specificities, as they reacted only with their homologous proteins, whereas antiserum against MP^{SABYV} reacted with all three viral proteins. Interestingly, we distinguished three different viruses by the strength of the serological response under these conditions; the strongest response was MP^{SABYV}, followed by MP^{MABYV}, and the weakest response was MP^{CABYV} (Fig. 4c). MP^{CABYV}, MP^{MABYV} and MP^{SABYV} share a high degree of homology. At the nucleotide level, MP^{SABYV} shares approximately 79.7% identity with MP^{CABYV}, and 88.5% identity with MP^{MABYV}. At the amino acid level,

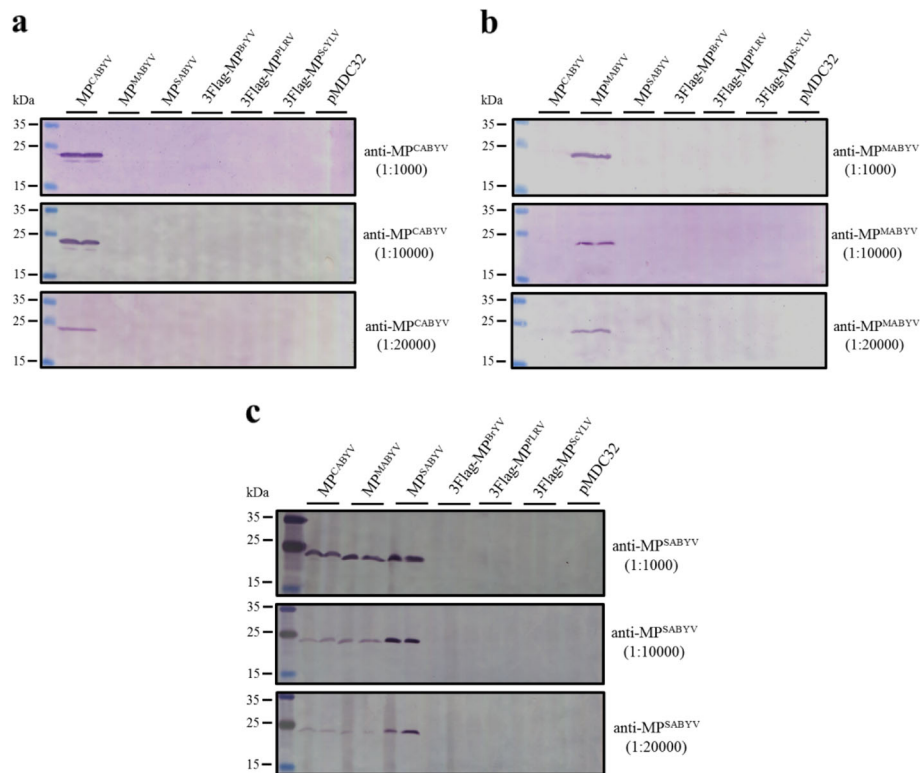


Fig. 4 Specificity analysis of antisera by western blotting. The left lane is the pageruler prestained protein ladder marker, and the other lanes are proteins extracted from *N. benthamiana* leaves, transiently expressing different MPs including CABYV, MABYV, SABYV, BrYV, PLRV and ScLYV. pMDC32 empty vector is a negative control. The three test antisera, anti-Mp^{CABYV} (a), anti-Mp^{MABYV} (b) and anti-Mp^{SABYV} (c) were used at 1:1000, 1:10000 and 1:20000

MP^{SABYV} shares only 65.4% identity with MP^{CABYV} and 75.1% identity with MP^{MABYV}, which is positively correlated with the strength of the serological response. However, we could not distinguish among these three viruses under the condition of low SABYV titer, and this issue should be addressed in the future research.

Cucurbit crops such as cucumber, watermelon, melon, and pumpkin are natural hosts of CABYV, MABYV and SABYV. The antisera we developed are not only ideal for MP detection in *N. benthamiana*, but also in natural hosts. The simulated detection results suggested that total proteins from cucumber did not interfere with the specific reactions between antisera and their corresponding MPs (Fig. 5). Equally, CABYV was detected in the natural host, passion fruit, by our antiserum against MP^{CABYV}, providing new evidence for the application of our antiserum in the field (Zhang et al. 2019). Our work shows that all antisera have a wide range of applications, although detection in cucurbits requires further verification.

It was reported that antiserum developed based on the MP from BYDV-PAV Qinghai isolate not only reacted with PAV105 isolate sharing 79.3% identity in amino acid, but also had strong serological cross-reactivity with the other BYDV MPs sharing 62.7–83.3% identity in amino acid (Hu et al. 2020), indicating that serological cross-reactions

existed between MPs with high homology. Previous studies showed that besides CABYV, MABYV, and SABYV, several other poleroviruses were also found infecting cucurbit crops, including luffa aphid-borne yellows virus (LABYV), pepo aphid-borne yellows virus (PABYV), and cucumber aphid-borne yellows virus (CuABYV) (Knierim et al. 2015). Additionally, zucchini aphid-borne yellows virus (ZABYV) was reported as a new virus in China in 2019 (Peng et al. 2019). MPs from these viruses share approximately 23.08–75.92% amino acid identity with MPs from CABYV, MABYV, and SABYV (Table 1), of which PABYV-MP showed the highest identity, and LABYV-MP showed the lowest identity. Further studies are required to investigate serological relationships among MPs of all these viruses.

Conclusions

CABYV, MABYV and SABYV are important viruses infecting cucurbit crops. The development of specific antisera is essential for virus detection, and exploring viral replication, movement and interaction with host plants. In our study, we expressed and purified three MPs using a prokaryotic expression system, and developed their polyclonal antisera of high titer, sensitivity and specificity, although antiserum against MP^{SABYV} had a relatively weaker reaction to MP^{CABYV} and MP^{MABYV}. The system

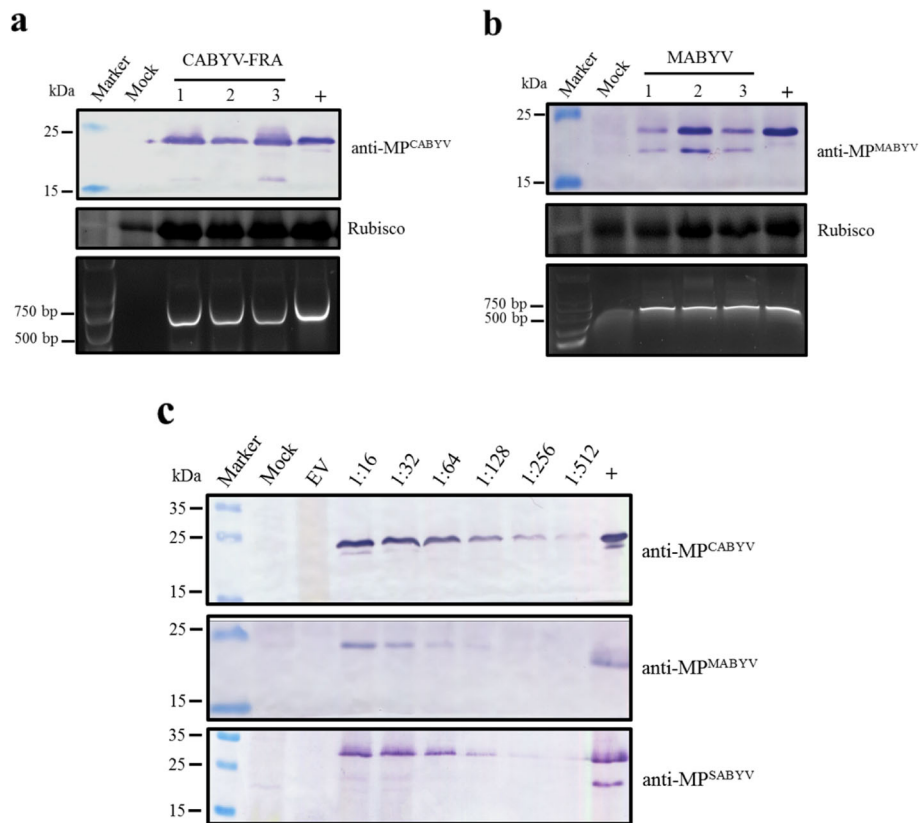


Fig. 5 Application analysis of antisera by western blotting. **a, b** Expression comparisons of CABYV and MABYV in *N. benthamiana* leaves inoculated with full-length infectious clones by western blotting and RT-PCR. Marker: pageruler prestained protein ladder; Mock: inoculated pCass-RZ empty vector; Lanes 1, 2, 3: leaf samples expressing CABYV or MABYV infectious clone; "+": proteins extracted from *N. benthamiana* leaves transiently expressing MP^{CABYV} or MP^{MABYV}. Rubisco stained with coomassie brilliant blue is used as a loading control (middle panel). The lower panels are RT-PCR data from the same samples. **c** Simulation detection of cucumber samples infected with CABYV, MABYV or SABYV. Marker: pageruler prestained protein ladder; Mock: proteins extracted from healthy cucumber leaves; EV: *N. benthamiana* leaves inoculated with pMDC32 empty vector; "+": protein extracted from *N. benthamiana* leaves transiently expressing the three MP genes; The other lanes show different dilutions of proteins extracted from *N. benthamiana* leaves transiently expressing MPs (1:16, 1:32, 1:64, 1:128, 1:256 and 1:512) and mixed with proteins extracted from healthy cucumber leaves. The three antisera were used at 1:10000

we established can be successfully used for virus serological detection, and the investigation of interactions between viruses and host plants.

Methods

Plant materials and growth conditions

N. benthamiana seeds were donated by Prof. David Baulcombe (Department of Plant Sciences, University of

Cambridge, UK), and were propagated and preserved in our laboratory. *N. benthamiana* plants were grown at 24 ± 1 °C and a relative humidity of 40%, with a 16-h light /8-h dark cycle.

Vector construction

Full-length MP^{CABYV} (Accession No: EU000535), MP^{MABYV} (Accession No: EU000534), and MP^{SABYV} (Accession No: NC_018571.2) genes were amplified by PCR using forward and reverse primers (Additional file 2: Table S1). The full-length cDNA clones pCaCABYV65 and pCaMABYV36 used as templates were constructed in our laboratory (Xiang 2011). The plasmid pMD19-SABYV containing ORF4 used as the template was synthesized by a biotech company (Tsingke biological technology, China). PCR was performed using Primer Star Max HS DNA Polymerase (TaKaRa, Japan) as previously described with slight modifications (Zhang et al. 2015).

Table 1 Comparison of MP amino acid sequence identity (%) between CABYV, MABYV, SABYV and other poleroviruses infecting cucurbits

Poleroviruses	Accession uumber	CABYV	MABYV	SABYV
LABYV	NC_027703	24.27	26.83	23.08
PABYV	NC_030225	75.92	70.31	71.88
CuABYV	FJ460218	59.28	59.28	60.31
ZABYV	MK050791	56.48	55.44	55.96

PCR products were electrophoresed on a 1% agarose gel, excised, purified and ligated into the prokaryotic expression vector pDB-His-MBP (DNASU Plasmid Repository, Arizona, USA), pre-digested with the restriction enzymes, *NdeI* and *XhoI* at 37 °C for 4 h. Histidine and MBP tags can increase protein solubility with no discernible effects on antigenic determinants of the recombinant protein. The ligation products were transformed into the *E.coli* strain, MC1022 to obtain the positive clones and extracted plasmids. The correctness of the extracted plasmids was verified by sequencing analysis (Tsingke biological technology, China). Transient expression vector pMDC32-MP^{CABYV}, pMDC32-MP^{MABYV} and pMDC32-MP^{SABYV} were constructed using the same methods (Curtis and Grossniklaus 2003).

Agrobacterium-mediated transformation

The freezing-thawing method with liquid nitrogen was used to transform the transient expression vectors into competent cells of *Agrobacterium tumefaciens* strain, GV3101 (Holsters et al. 1978). Approximately 3–5 µL plasmid was added to 100 µL of competent cells, gently mixed and incubated on ice for 30 min, and then frozen in liquid nitrogen for 1 min. The mix was melted at 37 °C for 5 min, after which 400 µL of LB liquid medium was added. After 4 h resuscitative mixing at 28 °C, the mix was spread onto LB plates supplemented with Kanamycin (50 mg/mL) and Rifampicin (50 mg/mL) and incubated at 28 °C for 48 h.

The agro-infiltration assays were performed as previously described (Zhuo et al. 2014; Zhang et al. 2018). *A. tumefaciens* strain GV3101 culture containing relevant vectors were individually incubated at 28 °C for 16 h, and mixed and injected into leaves of six-week-old *N. benthamiana* plants. All experiments shown in the results were repeated three times.

Recombinant protein purification and generation of polyclonal antisera

Three prokaryotic expression vectors carrying MPs, PDB-MP^{CABYV}, PDB-MP^{MABYV} and PDB-MP^{SABYV}, were individually transformed into the *E.coli* strain, *Rosetta*. Positive colonies were incubated overnight at 37 °C with shaking at 220 rpm in 10 mL of LB liquid medium containing kanamycin (50 mg/mL) and chloramphenicol (34 mg/mL), which were transferred to 1 L of LB liquid medium supplemented with kanamycin and chloramphenicol, and incubated at 37 °C with shaking for 4–6 h to generate an OD₆₀₀ between 0.6 and 0.8. Then, cultures were induced by 0.1 mM isopropyl-β-D-thiogalactoside (IPTG; Sigma-Aldrich, St. Louis, MO, USA) at 18 °C, and 180 rpm for 18 h. After that, bacteria were collected by centrifugation at 5000 rpm for 6 min. After ultrasonic disruption, supernatants were collected by

centrifugation at 16000 rpm for 40 min. Clarified supernatants were added to a nickel-affinity column (Qiagen, Hilden, Germany), and proteins were washed by different concentrations (20, 40, 60, 80, 100, 200, and 500 mM) of imidazole eluent. Each elution fraction was subjected to SDS-PAGE to assess MP expression. The fraction containing MP was concentrated to generate purified fusion protein. The molecular weights of three recombinant proteins were predicted by SnapGene Viewer software (SnapGene, USA).

New Zealand white rabbits were injected with 400 µg purified protein. After 2 weeks, rabbits were further given three consecutive enhanced immunizations (200 µg purified proteins), each 10 days apart. When immunizations were completed, positive blood was taken from the carotid artery, after which it was centrifuged twice at 5000 rpm for 10 min. From this, 30 mL polyclonal antisera against MP^{CABYV}, MP^{MABYV} and MP^{SABYV} were obtained (Beijing Protein Innovation Co. Ltd. conducted the rabbit immunization).

Protein extraction and western blotting

Protein extraction and western blotting were performed as described previously (Zhuo et al. 2014; Li et al. 2019). 0.1 g *N. benthamiana* leaf samples were ground into powder in liquid nitrogen and then added with 300 µL of 2 × SDS buffer (100 mM Tris (pH 6.8), 20% glycerol, 4% SDS, and 0.2% bromophenol blue) containing 10% β-mercaptoethanol. Each sample was used after heating at 100 °C for 10 min.

Proteins were separated by SDS-PAGE, and then transferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) by electro-transfer (200 mA, 100 min). Membranes were blocked in 1 × TBST buffer (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH = 8), containing 5% milk at 37 °C for 1 h, followed by incubation with polyclonal antisera against MP^{CABYV}, MP^{MABYV} and MP^{SABYV} respectively at 37 °C for 1 h. Subsequently, membranes were washed three times in 1 × TBST, incubated with alkaline phosphatase (AP)-labeled goat anti-rabbit IgG secondary antibody (1:20000, Easybio, China) at 37 °C for 1 h, and followed by washing three times in 1 × TBST. Then, membranes were incubated with buffer containing nitro-blue tetrazolium chloride (NBT) (330 µg/mL) and 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) (165 µg/mL) to reveal protein bands. The marker was pegeruler prestained protein ladder (Thermo, USA). Coomassie brilliant blue R250 was used to stain the gel to indicate equal loading (Bio-rad, USA).

Antisera titer determination

pMDC32-MP^{CABYV}, pMDC32-MP^{MABYV} and pMDC32-MP^{SABYV} (OD₆₀₀ = 0.5) were co-expressed with P19

(OD₆₀₀ = 0.2) in six-week-old *N. benthamiana* plants. Injection of the pMDC32 empty vector served as a negative control. P19 is a typical viral suppressor of RNA silencing (VSR) from tomato bushy stunt virus (TBSV) and the transient expression vector pGDP19^{TBSV} was provided by Prof. Andrew O. Jackson, University of California Berkeley (Bragg and Jackson 2004). Inoculated leaves were collected at 3 dpi for protein extraction. All three antisera were equally diluted 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000, 1:64000, 1:128000, 1:256000 and 1:512000 for western blotting to determine titers and optimal working concentrations.

Antisera sensitivity analysis

pMDC32-MP^{CABYV}, pMDC32-MP^{MABYV} and pMDC32-MP^{SABYV} (OD₆₀₀ = 0.5) were co-expressed with TBSV P19 (OD₆₀₀ = 0.2) in six-week-old *N. benthamiana* plants. The pMDC32 empty vector served as a negative control. Inoculated leaves were collected at 3 dpi to extract total proteins. Approximately 0.1 g *N. benthamiana* leaves were used to generate protein samples by adding 300 μL 2 × sodium dodecyl sulfate (SDS) buffer. Samples were diluted by two gradients (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024 and 1:40, 1:80, 1:160, 1:320, 1:640) for western blotting. Equally, all three antisera were diluted to 1:1000, 1:10000, and 1:20000.

Antisera specificity analysis

MP^{CABYV}, MP^{MABYV}, MP^{SABYV}, 3Flag-MP^{BrYV}, 3Flag-MP^{PLRV} and 3Flag-MP^{ScYLV} (OD₆₀₀ = 0.5) were co-expressed with TBSV P19 (OD₆₀₀ = 0.2) in six-week-old *N. benthamiana* plants. The pMDC32 empty vector served as a negative control (The three transient expression vectors, 3Flag-MP^{BrYV}, 3Flag-MP^{PLRV} and 3Flag-MP^{ScYLV} were provided by Deng-Pan Zuo in our laboratory). Inoculated leaves were collected at 3 dpi for total protein extraction. Western blotting was performed to assess antisera specificity. All three antisera were diluted to 1:1000, 1:10000 and 1:20000.

Detection of *N. benthamiana* inoculated with full-length infectious clones

We inoculated six-week-old *N. benthamiana* plants with full-length infectious CABYV and MABYV clones (Prufer et al. 1995; Xiang 2011). Injection of the transient expression vectors pMDC32-MP^{CABYV} and pMDC32-MP^{MABYV} served as positive controls and the pCass-RZ empty vector served as a negative control. Inoculated leaves were harvested at 3 dpi. Western blotting was performed using antisera at 1:10000, which was compared with the RT-PCR detection results of the same samples. RT-PCR was performed as previously described (Chen et al. 2018).

Simulation detection of natural host plants infected with virus

Total proteins from six-week-old *N. benthamiana* leaves, expressing MP^{CABYV}, MP^{MABYV} and MP^{SABYV} were extracted. The protein from healthy cucumber leaves was extracted using the same method. Then, cucumber protein and each MP protein were mixed separately in equal volume, which was equally diluted at 1:16, 1:32, 1:64, 1:128, 1:256 and 1:512. The pMDC32 empty vector, and cucumber protein served as negative controls. *N. benthamiana* leaves expressing MP^{CABYV}, MP^{MABYV} and MP^{SABYV} served as positive controls. Western blotting was then conducted using antisera at 1:10000 to observe the serological reactions of CABYV, MABYV and SABYV in cucumber.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s42483-020-00065-8>.

Additional file 1: Figure S1. Schematic representation of the CABYV-CN genome. CABYV-CN is a positive-sense single-stranded RNA virus containing 5682 nucleotides, with seven ORFs encoding seven proteins. A small VPg is covalently bound to the 5' end of the genomic RNA and there is neither a 3'-terminal poly (A) tail nor a tRNA-like structure in its RNA. ORF4 is 576 bp (3539–4114 nt) in length, and its MP is expressed by subgenomic RNA.

Additional file 2: Table S1. Primers used for vector construction.

Abbreviations

BrYV: Brassica yellow virus; CABYV: Cucurbit aphid-borne yellows virus; CP: Coat protein; CuABYV: Cucumber aphid-borne yellows virus; dpi: Days post inoculation; ELISA: Enzyme-linked immunosorbent assay; IPTG: Isopropyl-β-D-thiogalactoside; LABYV: Luffa aphid-borne yellows virus; MABYV: Melon aphid-borne yellows virus; MP: Movement protein; ORF: Open reading frame; PABYV: Pepo aphid-borne yellows virus; PLRV: Potato leafroll virus; RT-PCR: Reverse transcription polymerase chain reaction; SABYV: Suakwa aphid-borne yellows virus; ScYLV: Sugarcane yellow leaf virus; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBST: Tris-buffered saline; TBSV: Tomato bushy stunt virus; VPg: Viral genome-linked protein; VSR: Viral suppressors of RNA silencing; ZABYV: Zucchini aphid-borne yellows virus

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Authors' contributions

CGH conceived the study and revised the manuscript critically. SKZ, XS and YZL designed and carried out the experiments. SKZ and TYZ drafted the manuscript. YW, ZYZ, DWL, JLY and QXS contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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