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Molecular characteristics and pathogenicity of infectious cDNA clones of the Chinese isolates of pea enation mosaic virus 1 and pea enation mosaic virus 2

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Abstract

The pea enation mosaic disease (PEMD) causes significant yield losses worldwide. PEMD is caused by two taxonomically unrelated but symbiotic viruses, pea enation mosaic virus 1 (PEMV-1) and pea enation mosaic virus 2 (PEMV-2). In this study, the complete genomes of four isolates of PEMV-1 and PEMV-2 from Yunnan Province of China were determined and analyzed. The four isolates of PEMV-1 or PEMV-2 shared ≥ 98.7% nucleotide sequence identities at whole genome level. The four Chinese PEMV-1 isolates were most closely related to PEMV-1-Ramsey-1 with nucleotide sequence identities of 97.8–98.0%, while the four Chinese PEMV-2 isolates were most closely related to PEMV-2-DSMZ PV-0088, with nucleotide sequence identities of 94.3–94.5%. The two full-length infectious cDNA clones of the Yunnan-Dali isolates of the two viruses (PEMV-1-YDL and PEMV-2-YDL) were constructed. Co-inoculation of PEMV-1-YDL and PEMV-2-YDL in pea seedlings caused systemic infection, with typical enation and mosaic symptoms on new leaves after inoculation, while the inoculated *Nicotiana benthamiana* plants showed curling symptoms. Inoculation of pea seedlings with either PEMV-1-YDL or PEMV-2-YDL did not cause obvious symptoms on the new leaves. It has been known that PEMV-2 helps the systemic infection of PEMV-1. However, our results showed that PEMV-1-YDL alone could cause a systemic infection. The pathogenicity of the two infectious clones was tested on different pea cultivars, and some cultivars were PEMD-tolerant. This is the first report of the infectious clones constructed from the Chinese isolates of PEMV-1 and PEMV-2. The current approach will facilitate determination of the synergistic relationship between PEMV-1 and PEMV-2 and their interactions with the host plant by reverse genetic analysis.

Keywords Pea enation mosaic virus 1, Pea enation mosaic virus 2, Infectious cDNA clone, Resistance screening, Pathogenicity

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Background

Pea (*Pisum sativum* L.) is an edible legume and can be used as grain, vegetable, fodder, and green manure. It has become an economically important crop in dietary structure for human health and sustainable agriculture over the last 50 years due to its nutritional value and biological nitrogen fixation (FAOSTAT, http://www.fao.org/faostat/en/#data). Pea seeds are excellent sources of protein, dietary fiber and mineral nutrients so they are critical staple food, especially in developing countries. As a cool season legume, planting of the crop in winter or early spring is generally rotated with cereals, providing essential nitrogen and other nutrients to the soil for the following crops. Pathogens including viruses usually cause serious diseases in pea due to limited disease resistance and changes in cultivation and environment (Swanson and MacFarlane 1999; Sarkisova et al. 2016; Fowkes et al. 2021).

Pea enation mosaic disease (PEMD) is an important viral disease caused by two distinct viruses, pea enation mosaic virus 1 (PEMV-1, genus *Enamovirus* of family *Solemoviridae*) (Demler et al. 1991) and pea enation mosaic virus 2 (PEMV-2, genus *Umbravirus* of family *Tombusviridae*) (Demler et al. 1993), which are engaged in an obligate symbiosis. The pathogen was initially considered to be a bipartite virus with a genome of two positive-sense ssRNAs (Hull and Lane 1973), but subsequent studies showed that pea enation mosaic virus (PEMV) was a more stable version of an enamovirus helper-dependent virus complex (Demler et al. 1996). The interdependent virus complex is referred to as PEMV in this study unless the information specifically refers to one of the two viruses. PEMV causes various symptoms including typical vein enations, plant growth reduction and pod or seed deformities that cause significant yield losses. PEMV is transmitted either by mechanical inoculation or aphids, mainly pea aphids, in a circulative, nonpropagative manner, but seed transmission is not defined (Demler et al. 1997; Timmerman-Vaughan et al. 2009). The PEMV has been reported in many countries such as the USA, Canada, Germany, Spain, Syria, and China (Makkouk et al. 1999; Golnaraghi et al. 2004; Tornos et al. 2008; Vemulapati et al. 2010; Chen et al. 2021). In 2019, infection of peas by PEMV and severe yield losses were first reported in Yunnan, China (Chen et al. 2021). The genome of PEMV-1 contains five main open reading frames (ORFs). The first ORF (ORF 0) encodes a protein of 34 kDa, and ORF 1 a protein of 84-kDa. ORF 2 encodes an RNA polymerase of 67 kDa. ORF 3 encodes a coat protein (CP) of 21-kDa, which is immediately followed by ORF 5 encoding a 33-kDa protein expressed as a fusion with the 21 kDa CP, resulting in a read-through protein (RTP) of 54 kDa that is associated with aphid transmission (Demler et al. 1997). The PEMV-2 genome is typical of umbraviruses, consisting of four ORFs that encode proteins of various functions. ORF 1 encodes a putative 33-kDa protein of unknown function, and ORF 2 condes for a protein with motifs of a viral RNA-dependent RNA-polymerase (RdRp). ORFs 3 and 4 potentially encode 26 kDa and 27 kDa proteins which guide virus cell-to-cell and systemic movement, respectively (Demler et al. 1993, 1994a; Vemulapati et al. 2011; May et al. 2020). Both PEMV RNAs code for their respective RdRp and replicate independently in cells. PEMV-2 infects the entire plant but lacks the coat protein. Hence, it depends on PEMV-1 for its coat protein and vector-transmission functions (Demler et al. 1997). PEMV-2, in turn, provides the functions related to systemic movement and mechanical transmission (Demler et al. 1997). Earlier studies on PEMV revealed the presence of a third, smaller RNA (RNA-3) in some virus isolates (Demler and de Zoeten 1989; Demler et al. 1994b). RNA-3 is neither infectious on its own nor essential for the infectivity of PEMV-l and PEMV-2 (de Zoeten et al. 2001).

Generally, infectious clones are constructed through two strategies, in vitro viral RNA transcripts (Van Der Werf et al. 1986; Janda et al. 1987) and in vivo viral RNA transcripts (Odell et al. 1985; Grimsley et al. 1986). The in vitro transcript strategy was first used to construct infectious cDNA clones of brome mosaic virus (Ahlquist et al. 1984), and then the infectious clones of many plant viruses including PEMV-1 and PEMV-2 (Demler et al. 1993, 1994a). Infections of the cDNAs of PEMV-1 and PEMV-2 genomic components showed that PEMV-1 replicated alone in protoplasts but not in intact plants, while PEMV-2 replicated alone in both protoplasts and plants. Under the control of the CaMV 35S promoter via agro-infiltration assays, in vivo viral RNA transcripts have also been produced from the infectious clones of many viruses such as beet western yellows virus, potato leafroll virus (PLRV), barley yellow dwarf virus-PAV, cereal yellow dwarf virus-RPV, and Brassica yellows virus (BrYV) and used for their infections of numerous plants (Leiser et al. 1992; Kawchuk et al. 2002; Yoon et al. 2011; Zhang et al. 2015). PEMV-2 infectious cDNA clone under the control of a duplicated CaMV 35S promoter was reported recently (Zhou et al. 2017; May et al. 2020). This study showed that the synergism of PEMV-2 (LANS strain) and BrYV led to the appearance of severe symptoms in *Nicotiana benthamiana* plants. An increasing number of studies have been reported the application of infectious virus clones as powerful tools to investigate the arms race between viruses and their host plants (Wang et al. 2015; Gao et al. 2019; Feng et al. 2020; Ma et al. 2021). Additionally, virus-based vectors are used to screen

disease-resistant cultivars (Jin et al. 2012; Bang et al. 2014). Virus-based vectors are faster, more convenient and easier to quantify than viruses in investigation of the diseases in inoculated plants. Although the Agrobacterium-based infectious clone of PEMV-2 has been reported, the Agrobacterium-based infectious clone of PEMV-1 is still not available due to the particularity of this disease. Therefore, it is necessary to construct PEMV-1 and PEMV-2 of the same isolate to shed light on their synergetic relationship.

We report here the four full-length genomic sequences each of PEMV-1 and PEMV-2 from Yunnan Province, China for the first time. Infectious cDNA clones were constructed from the full-length PEMV-1-YDL and PEMV-2-YDL genomic sequences expressed downstream of the double 35S promoter ($2 \times 35S$) by homologous recombination. Results of RT-PCR using virus-specific primers indicated that PEMV-1-YDL alone caused a systemic infection. In addition, the infectivity of these two cDNA clones was further evaluated in different pea cultivars.

Results

Symptoms observation and detection of PEMV-1 and PEMV-2 in the pea plants

Symptoms of foliar mosaic and enation resembling those caused by PEMV were observed in the pea fields in Kunming City, Dali, and Honghe Autonomous Prefecture (Mengzi and Mile counties) of Yunnan Province of China in January 2020. In the pea field of Mile county, the diseased peas showed chlorosis in lower leaves, mosaic in upper leaves, color breaking in flowers and deformation in pods (Fig. 1a, no pod symptom). Forty-four samples were collected from the above four regions. These samples were detected for PEMV-1 and PEMV-2 by RT-PCR using PEMV-specific primers (Additional file 1: Table S1). Amplicons of 801 and 483 bp were obtained for PEMV-1 and PEMV-2, respectively, from all 44 samples (Fig. 1b–e). Three amplicons were randomly selected for each isolate for cloning and sequencing, and sequences of three amplicons were 100% identical for each isolate (Additional file 1: Table S2). The sequences of the 801-bp fragments of PEMV-1 shared the nucleotide (nt) sequence identities of 99.3–99.6% among the four Chinese PEMV-1 isolates. They shared the highest nt sequence identities (98.9–99.1%) with PEMV-1-PRTH-PEMV1-IDT (OK030718.1). The 483-bp fragments of PEMV-2 shared 99.2% nt sequence identities among the four Chinese PEMV-2 isolates. These amplicons had the highest nt sequence identities (97.6–98.0%) with the PEMV-2-Wainfleet (OK030734.1). Therefore, PEMV is associated with the pea diseases in the four regions of Yunnan, and the four isolates of both viruses each were very similar as for nucleotide sequences.

Sequence alignment and phylogenetic analysis of PEMV-1 and PEMV-2

The full-length genomes of four PEMV-1 and four PEMV-2 isolates infecting peas in Yunnan Province were determined and analyzed. The genomes of PEMV-1-YKM, PEMV-1-YML, and PEMV-1-YMZ are 5703 nts, while the genome sequence of PEMV-1-YDL comprises 5704 nts. The genome organization of all PEMV-1 isolates are identical to those of previously reported PEMV-1 isolates, containing five ORFs (Fig. 2a). Pairwise alignment of the nucleotide sequences revealed very high sequence identities (99.2–99.5%) among the four Chinese PEMV-1 isolates. They shared more than 95.0%

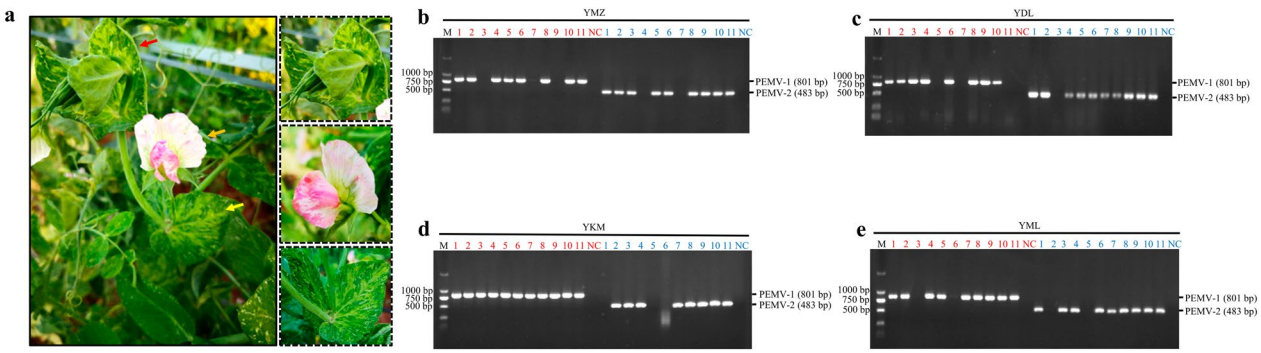

Fig. 1 Symptoms and RT-PCR detections of PEMV in diseased pea plants. **a** Symptoms observed on PEMV-infected pea plants in Mile of Yunnan Province. Red, orange, and yellow arrows indicate mosaic, flower broken, and chlorosis, respectively. The dashed rectangle represents in the different shooting angles of the left plants. **b**–**e** Eleven samples were collected from Mengzi (YMZ), Dali (YDL), Kunming (YKM) and Mile (YML) of Yunnan, and tested by RT-PCR using specific primers for PEMV-1 (801 bp) and PEMV-2 (483 bp). Lane M, DL2000 DNA marker (TaKaRa Biotechnology, Dalian, China); Lanes 1–11, the amplified PCR products using primers for PEMV-1 (in red font) and PEMV-2 (in blue font); NC, the negative control. The same number in each figure represents the same plant sample

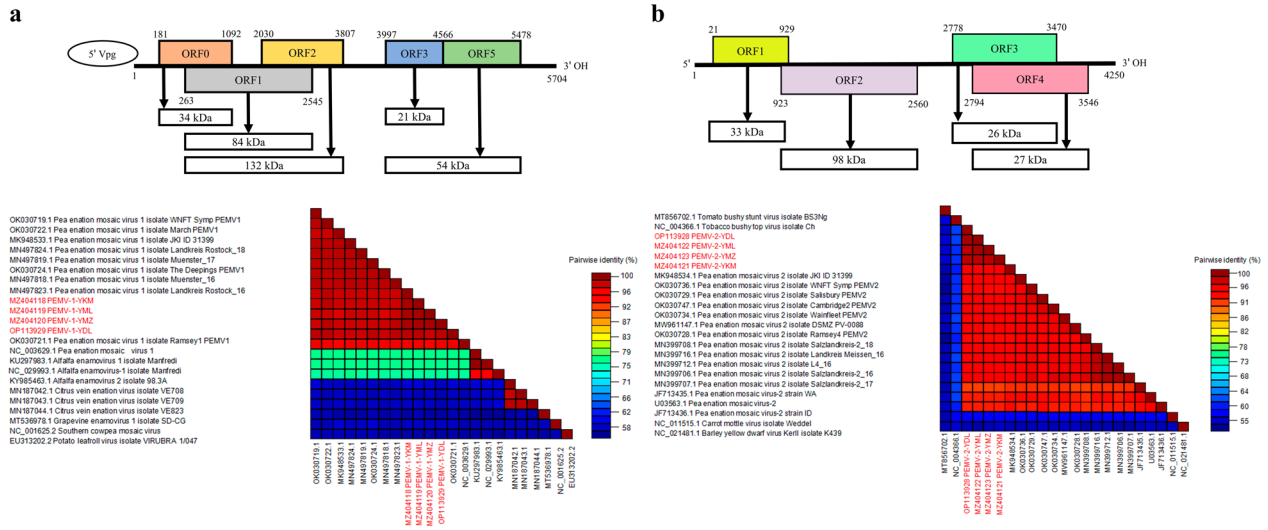

Fig. 2 Genome organization of PEMV-1 and PEMV-2, and pairwise identity matrices of the full-length genome sequences generated by the Sequence Demarcation Tool version 1.2. **a** Genome organization of PEMV-1 and pairwise identity matrix of PEMV-1 and other viruses in the genus *Enamovirus*, *Polerovirus*, and *Sobemovirus* in the family *Solemoviridae*. **b** Genome organization of PEMV-2 and pairwise identity matrix of PEMV-2 and other viruses in the genera *Umbravirus*, *Tombusvirus*, and *Luteovirus* of the family *Tombusviridae*

nt sequence identities with other PEMV-1 isolates available in the GenBank database. Besides, PEMV-1-YDL, PEMV-1-YML, and PEMV-1-YMZ shared the highest nt sequence identities (98.0, 98.0, and 97.8%) with two PEMV-1 isolates from the United Kingdom (PEMV-1-Ramsey-1, OK030721.1, and PEMV-1-The-Deepings, OK030724.1). PEMV-1-YKM shared 97.9% and 98.0% nt sequence identities with PEMV-1-Ramsey-1 and PEMV-1-The-Deepings, respectively. The four Chinese PEMV-1 isolates shared slightly lower nt sequence identities (96.1–96.4%) with PEMV-1 isolate from the United States (NC_003629.1), and 48.8–76.4% nt sequence identities with other reported enamoviruses, and only 42.8–46.1% nt sequence similarities with certain viruses in the genera *Polerovirus* and *Sobemovirus* of the family *Solemoviridae* (Fig. 2a).

The genomes of all PEMV-2 isolates comprised 4250 nts with four predicted ORFs (Fig. 2b). The pairwise alignment showed 98.7–99.0% of nt sequence identities in this region among the four Chinese isolates of PEMV-2. The four Chinese PEMV-2 isolates shared more than 90.3% nt sequence identity with other PEMV-2 isolates available in the GenBank. PEMV-1-YDL, PEMV-1-YML, PEMV-1-YMZ, and PEMV-1-YKM shared the highest nt sequence of 94.3–94.5% with two PEMV-2 isolates from Germany (PEMV-2-DSMZ PV-0088, MW961147.1 and PEMV-2-Salzlandkreis-2_17, MN399707.1). The four Chinese isolates of PEMV-2 shared relatively lower nt sequence similarities 90.3–90.4% with the American isolate of PEMV-2 (JF713435.1). The four Chinese PEMV-2 isolates shared 54.1–56.1% nt sequence identities with other reported umbraviruses and 42.2–47.5% nt sequence identities with certain viruses in the genus *Tombsuvirus* or *Luteovirus*, family *Tombusviridae* (Fig. 2b).

Phylogenetic trees were constructed using the nt sequences of four PEMV-1 and four PEMV-2 isolates from the Yunnan Province and those retrieved from the GenBank, respectively (Fig. 3). The four Chinese PEMV-1 isolates clustered on a branch and grouped in the same cluster with previously reported PEMV-1 isolates, as expected from their high sequence identities. Furthermore, they were most closely related to PEMV-1-Ramsey-1 (Fig. 3a). The four Chinese isolates and other available isolates of PEMV-2 also grouped in a cluster that was separated from other viruses in the genera *Umbravirus*, *Tombsuvirus*, and *Luteovirus* of the family *Tombusviridae* (Fig. 3b).

Infectivity and pathogenicity of the PEMV-1 and PEMV-2 infectious clones

The full-length genome sequences of the PEMV-1-YDL and PEMV-2-YDL isolates were cloned into the binary vector pCB301 under the control of the 35S promoter of a cauliflower mosaic virus using a one-step assembly strategy (PEMV-1-YDL and PEMV-2-YDL) (Fig. 4a). The infectivity and pathogenicity of the two viruses in the pea plants were examined by inoculating the viral infectious clones either individually or in combination through agro-infiltration. At 7 days post-infection (dpi), pea plants co-inoculated with both viruses showed

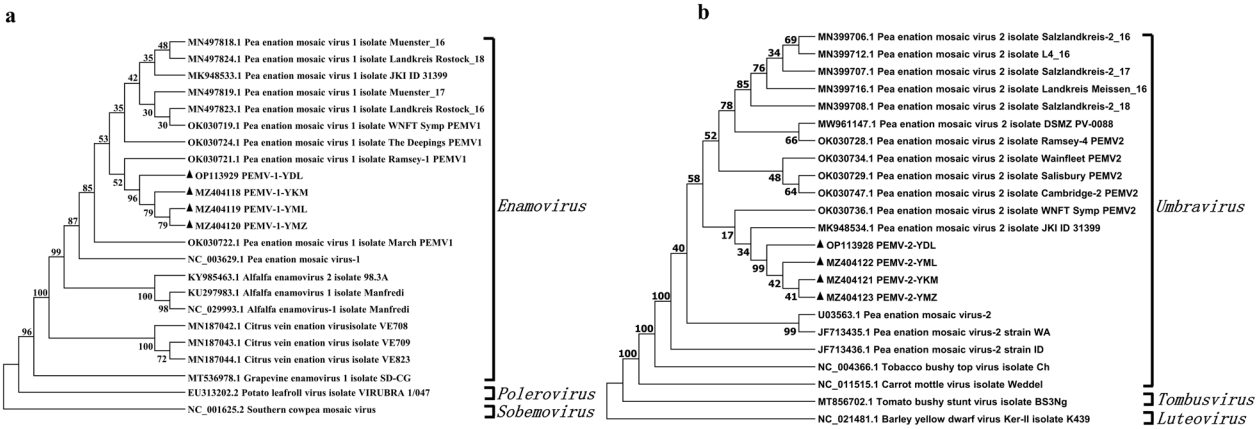

Fig. 3 Construction of the Neighbor-joining phylogenetic tree using the complete genome sequences of PEMV-1, PEMV-2, and other related viruses. The statistical significance of the branches was calculated by bootstrap with 1000 replicates. **a** Phylogenetic tree was constructed using the nucleotide sequences of PEMV-1 and other viruses in the 3 genera *Enamovirus*, *Polerovirus*, and *Sobemovirus*. **b** Phylogenetic tree was constructed using the nucleotide sequences of PEMV-2 and other viruses in the genera *Umbravirus*, *Tombusvirus*, and *Luteovirus*

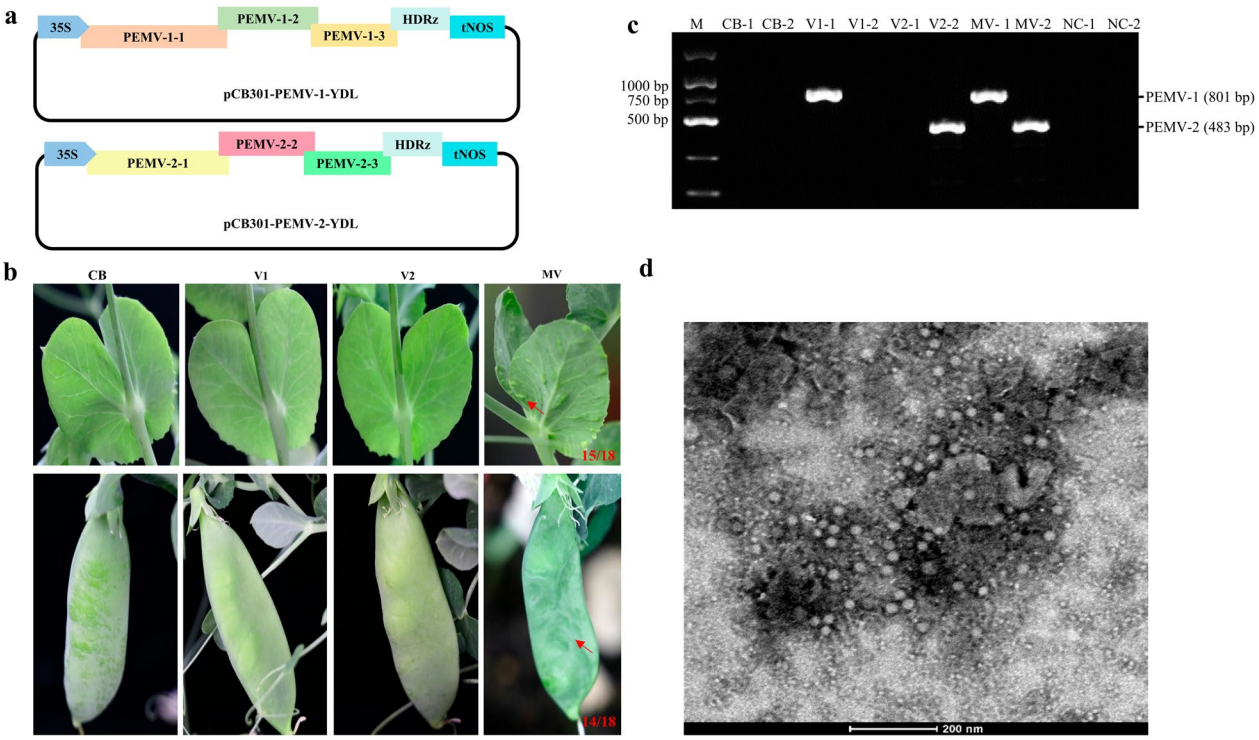

Fig. 4 Infectivity and pathogenicity of the infectious clones of PEMV-1-YDL and PEMV-2-YDL. **a** Schematic diagram illustrating the one-step assembly cloning strategy used to construct infectious full-length cDNA clones of PEMV-1-YDL and PEMV-2-YDL. Three overlapping fragments of PEMV-1-YDL and PEMV-2-YDL were amplified and inserted into the linearized pCB301 vector. 35S, the cauliflower mosaic virus promoter; HDRz, hepatitis delta virus ribozyme; tNos, Nos terminator. **b** Symptoms of systemic leaves and pods of pea (Taiwan Changshou) plants PEMV-1-YDL or PEMV-2-YDL or mixture of PEMV-1-YDL and PEMV-2-YDL using agro-infiltration. Red arrows indicated enations on the systemic leaves and pods. Pea plants and pods were photographed at 21 and 33 days post-infection (dpi), respectively. The plants were inoculated with Agrobacteria that harbor the pCB301 vector as a control. **c** RT-PCR detection of PEMV-1-YDL and PEMV-2-YDL in the inoculated pea at 21 dpi. '-1' and '-2' indicated RT-PCR detection using specific primers of PEMV-1 (801 bp) and PEMV-2 (483 bp). Lane M, DL 2000 DNA Marker; lanes CB, plants inoculated with pCB301; lanes V1, plants inoculated with PEMV-1-YDL; lanes V2, plants inoculated with PEMV-2-YDL; lanes MV, plants inoculated with PEMV-1-YDL+PEMV-2-YDL; lanes NC, represent negative control. **d** Transmission electron micrograph of viral particles in the systemic leaves of pea plants inoculated with PEMV-1-YDL+PEMV-2-YDL via agro-infiltration. Bar = 200 nm

mosaic in the new leaves, followed by the auricular protuberance of the leaf abaxial surface by 21 dpi and pods by 33 dpi. Additionally, plants inoculated with PEMV-1-YDL or PEMV-2-YDL did not show obvious symptoms on the systemic leaves (Fig. 4b). RT-PCR results showed the accumulation of both viruses in the systemic leaves of the plants co-inoculated with PEMV-1-YDL and PEMV-2-YDL. The pea plants inoculated with PEMV-1-YDL or PEMV-2-YDL revealed the presence of corresponding viruses in their systemic leaves through RT-PCR (Fig. 4c). Transmission electron microscopy of the crude extracts from the systemic leaves of the symptomatic pea plants inoculated with PEMV-1-YDL+PEMV-2-YDL revealed the presence of icosahedral viral particles of approximately 28 nm in diameter (Fig. 4d), which are the typical morphology of the family *Solemoviridae*. The *N. benthamiana* plants developed leaf-curling symptoms by 16 dpi after co-inoculation with PEMV-1-YDL and PEMV-2-YDL (Additional file 2: Figure S1a). Visible symptoms were not observed in the systemic leaves of the plants inoculated with either PEMV-1-YDL or PEMV-2-YDL although RT-PCR results showed the accumulation of PEMV-1-YDL and/or PEMV-2-YDL in the systemic leaves of the *N. benthamiana* plants inoculated with PEMV-1-YDL and/or PEMV-2-YDL was detected (Additional file 2: Figure S1b).

Screening of pea cultivars for resistance to PEMV

To investigate the resistance or tolerance of cultivated cultivars to PEMV, seven commercially cultivated pea

Fig. 5 Symptoms in the systemic leaves of different cultivars of pea plants inoculated either alone or in various combinations by PEMV-1-YDL and/or PEMV-2-YDL using agro-infiltration. Red, orange, and yellow arrows indicate enations, chlorosis, and necrotic spots in the systemically infected leaves and pods. Images were taken at 21 days post-infection (dpi) for pea plants and 33 dpi for pea pods. The plants were inoculated with Agrobacteria that harbor the pCB301 vector as a control

Table 1 Symptoms and infection rates of the infectious clones of PEMV-1-YDL+PEMV-2-YDL on different pea cultivars

Pea cultivars	Systemic leaf symptoms	Systematic leaf incidence (%)	Pea pod incidence (%)	PEMV-1-YDL infection rate (%)	PEMV-2-YDL infection rate (%)	PEMV-1-YDL+PEMV-2-YDL infection rate (%)
Qizhen 76	Enations, chlorosis, necrosis	16.7	11.1	44.4	94.4	44.4
Taiwan Langchao 2	Enations, chlorosis	25.0	0	75.0	95.8	75.0
Lvsheng no vine	Enations, necrosis	30.8	30.8	76.9	92.3	69.2
Jingxuan	Enations, chlorosis, necrosis	62.5	50.0	93.8	81.3	75.0
Pink flower pod	Enations, chlorosis, mosaics	66.7	52.4	81.0	90.5	76.2
Taiwan Changshou	Enations	83.3	77.8	88.9	100	88.9
Xishouren 1	Enations	90.9	72.7	100	100	100

cultivars from Yunnan Province were challenged with PEMV. Most pea seedlings exhibited typical enation mosaic symptoms at 21 dpi (Figs. 4b, 5). The pathogenic rates ranged from 16.7% to 90.9% in the seven cultivars (Table 1). The RT-PCR results showed the infection rates of PEMV was 100.0% on Xishouren 1, indicating the variety was most susceptible to the viruses. The lowest infection rate of 44.4% occurred on Qizhen 76, suggesting it had certain degree of resistance. The infection rates on the other cultivars ranged from 69.2% to 88.9% (Table 1).

Discussion

Pea is widely grown in temperate regions, and its cultivation in the tropics is restricted to the cold season. It is widely planted as vegetables (leaves and young pods) and staple food (seeds) in Yunnan Province. PEMV is a problem in most the pea growing areas of the United States and Germany (Larsen 2010; Gaafar et al. 2020). Both PEMV-1 and PEMV-2 were found recently to be associated with diseases of the pea plants in the Honghe county of Yunnan for the first time in China (Chen et al. 2021). A recent study showed the prevalence of the PEMV-1, PEMV-2, and pea necrotic yellow dwarf virus in pea plants throughout Germany but not in every season (Gaafar et al. 2020). In our study, the PEMV outbreak was discovered in many regions of Yunnan, almost throughout the year, probably due to the mild climate in these regions.

PEMV was first described and characterized in 1935 in the faba beans (Makkouk et al. 2012) and have been reported in many countries such as Syria, the USA, Spain, and Germany. In China, PEMV was first reported in pea plants in the Honghe Autonomous Prefecture of Yunnan Province in 2019 (Chen et al. 2021). We surveyed many pea planting regions in Yunnan, and PEMV was identified in Dali region, Kunming city, Mengzi and Mile counties of Honghe region. To better understand the genetic diversity of these two viruses, the complete genome sequences of four isolates were determined for both viruses, respectively. Compared to PEMV-1, the four Chinese PEMV-2 showed more notable similarities and differences from other isolates. Phylogenetic analyses of the nucleotide sequences from the genome sequences of PEMV-1 and PEMV-2 revealed that the Chinese isolates clustered as a separate group from the remaining isolates.

The genomic structure of PEMV-1 is different from other viruses of the family *Solemoviridae* in lacking ORF 4 that encodes a putative movement protein. This protein is necessary for systemic infection in plants. Therefore, PEMV-1 depends on PEMV-2 for mechanical inoculation and systemic movement (Demler et al. 1993, 1996). This study demonstrated systemic invasion by PEMV-1-YDL beyond the inoculated leaves by agro-infiltration. Thus, PEMV-1-YDL does not require the assistance of PEMV-2 for systemic movement. Considering that the systemic movement of PEMV-1-YDL is a function of the PEMV-1-coded proteins, identification of the PEMV-1 protein/proteins involved in the systemic and non-systemic movement will be useful in controlling the systemic spread of the viruses. The mutations in the CP clearly affect the long-distance movement of PEMV-1 and PEMV-2 (Doumayrou et al. 2016). The CP and RTP also affect systemic infection of PLRV (Lee et al. 2005; Xu et al. 2018). In this study, based on the amino acid sequence analysis, PEMV-1 has the CP and RTP domains that are highly conserved in PLRV. The amino acid sequences of the CP and RTP had low variability among different isolates of PEMV-1 (Additional file 1: Table S3). There were three different amino acid residues at positions 19 V/A, 25A/S, and 40N/S in CP and twelve different amino acid residues at positions 43 V/I, 118A/S, 140S/Y, 141A/T, 172R/H, 188D/E, 254A/V, 257P/L, 259L/F, 264 V/A, 275G/D, and 282R/K in RTP between PEMV-1-YDL and PEMV-1-AT+(GenBank accession number Y09099.1) (Additional file 2: Figure

S2). It is likely that these amino acid changes in CP and RTP, which are known to be important for virus long-distance movement, influenced the ability of the viruses to cause systemic infections. Furthermore, there may be other PEMV-1 proteins involving in long-distance movement but have not yet been found since non-AUG-initiated ORF3a is required for long-distance movement for poleroviruses and luteoviruses (Smirnova et al. 2015). As for the Czech PEMV isolates, phylogenetic analyses of the genes encoding the PEMV-1 CP and the PEMV-2 MP demonstrated differences in the evolution of PEMV-1 and PEMV-2 (Šafářová and Navrátil 2014). However, whether these differences affect the PEMV-1 long-distance movement is not mentioned by the authors. In addition, both in vitro and in vivo RNA transcripts are used to construct infectious cDNA clones as mentioned above. PEMV-1-YDL in this study can establish systemic infection, this would be caused by the more efficient infection to the vascular system by Agrobacterium-mediated infectious clone of PEMV-1, which has not been developed previously.

Umbraviruses such as carrot mottle virus have been reported to enhance the symptom severity and accumulation of various poleroviruses by facilitating translocation between the phloem and mesophyll cells (Mayo et al. 2000; Ryabov et al. 2001; Zhou et al. 2017). PEMV-1 or PEMV-2 could cause systemic infection, but they do not show visible symptoms. Only double infections of PEMV-1 and PEMV-2 induce typical enation and mosaic symptoms. The invasion of PEMV-1 into the mesophyll cells and/or the over-accumulation of PEMV-1 in the vascular tissue causes notable changes in the symptoms of double infections. The symptoms of PEMV-inoculated *N. benthamiana* are different from those found in pea plants. This observation was consistent with previous reports, in which no enations were observed in any experimental host. *N. tabacum* cv. White Burley remains asymptomatic when infected by PEMV (Motoyoshi et al. 1974). Therefore, the symptoms of PEMV are host-specific.

Resistance to PEMV has been a fundamental goal of green pea breeding for many years (Gaafar et al. 2020). Resistance or partial resistance to PEMV has been identified in dry land legume crops such as pea (Makkouk et al. 2014) and lentils (Aydin et al. 1987). However, identification of resistant or tolerant cultivars is still the focus of future work. The earlier standard methods to screen resistant cultivar is by inoculating pea tissue with PEMV in vitro transcripts or PEMV-acquired aphids (Aydin et al. 1987; Larsen and Porter et al. 2010). In comparison, the cost of Agrobacterium-mediated inoculation is lower and the efficiency of it is higher. Some susceptible cultivars had an infection rate of as high as 100% (Table 1). At the same time, the infection rates of PEMV-1 in most cultivars were lower than that of PEMV-2, indicating a certain tolerance of these cultivars to PEMV-1. However, no resistance has been observed for PEMV-2. In other words, PEMV-2 exhibited stronger infectivity.

Conclusions

In this study, we characterized four isolates of each of PEMV-1 and PEMV-2 infecting the pea plants in Yunnan Province. Sequence analyses revealed more than 98.7% nucleotide identities among the isolates of each virus. The Chinese isolates of PEMV-1 and PEMV-2 were most closely related to a British isolate (PEMV-1-Ramsey-1) and a German isolate (PEMV-2-DSMZ PV-0088), respectively. Two full-length infectious cDNA clones were constructed and characterized. Our results indicated that PEMV-1-YDL alone could cause a systemic infection, which was different from previous study. Use of the infectious clones is effective for screening resistant resources, and several commercial pea cultivars were screened. The construction and characterization of the PEMV-1 and PEMV-2 infectious clones will allow for further study of the synergistic relationship between PEMV-1 and PEMV-2, and their interactions with the host plants.

Methods

Virus source, plant materials, and growth conditions

Pea plants showing typical PEMV symptoms were collected from regions of Dali, Kunming, Mengzi, and Mile in Yunnan Province, China. The isolates of PEMV-1 and PEMV-2 from these regions were named PEMV-1-YMZ (MZ404120), PEMV-1-YDL (OP113929), PEMV-1-YKM (MZ404118), PEMV-1-YML (MZ404119), PEMV-2-YMZ (MZ404123), PEMV-2-YDL (OP113928), PEMV-2-YKM (MZ404121), and PEMV-2-YML (MZ404122), respectively. Seven pea cultivars (Qizhen 76, Taiwan Langchao 2, Lvsheng no vine, Jingxuan, Pink flower pod, Taiwan Changshou, Xishouren 1) were collected from local markets. Pea and *N. benthamiana* plants used for virus inoculation were grown in an insect-free greenhouse with 22 ± 1°C and 16-h light/8-h dark photoperiod.

RNA extraction, cDNA synthesis, and virus detection

Total RNA was extracted from symptomatic leaves using the TRIzol reagent according to the manufacturer's protocol (TIANGEN Biotechnology, Beijing, China). First-strand cDNA was synthesized from 1 μg of total RNA in a total volume of 10 μL by the Reverse Transcriptase M-MLV (RNaseH) and random primers (Takara

Biotechnology, Dalian, China) according to the manufacturer's instructions. The cDNA was stored at −20°C. The primer pairs PEMV-1-782-F/PEMV-1-1582-R and PEMV-2-2983-F/PEMV-2-3465-R were used for the RT-PCR detection of PEMV-1 and PEMV-2, respectively. These two primer pairs amplified DNA fragments of 801 bp (PEMV-1) and 483 bp (PEMV-2), respectively. The primer sequences are listed in Additional file 1: Table S1. The polymerase chain reaction (PCR) was performed using the 2X Taq Master Mix (Vazyme Biotech, Nanjing, China).

Complete genome amplification and sequencing

Based on the viral contigs identified by high-throughput RNA sequencing (Chen et al. 2021), primers were designed to amplify the full-length nucleotide sequences of the PEMV-1 and PEMV-2 isolates from Yunnan (China). Five pairs of primers (Additional file 1: Table S1) were used to amplify five overlapping fragments comprising the entire genome of PEMV-1. Another five primer pairs were designed to amplify adjacent regions with overlapping ends for PEMV-2 (Additional file 1: Table S1). RT-PCR was performed using the One Step TB Green® PrimeScript™ RT-PCR Kit as described by the manufacturer's instructions (TaKaRa Biotechnology, Dalian, China). The 5'-and 3'-terminal sequences of the PEMV-1 and PEMV-2 genome were determined using the SMARTER® RACE 5′/3′ (TaKaRa Biotechnology) using virus-specific (Additional file 1: Table S1) and RACE primers according to manufacturer's instructions. The PCR and RACE products were purified using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech, Shanghai, China) and cloned into the pMD19-T Vector (TaKaRa Biotechnology, Dalian, China) for the Sanger sequencing. Resulted sequences were assembled to the full-length genome sequences using the EditSeq software (Lasergene 7.0, DNASTAR Inc., USA).

Phylogenetic analysis

The whole genome sequences of the Chinese PEMV-1 and PEMV-2 isolates were used to BLAST search the GenBank database using the MUltiple Sequence Comparison by Log-Expectation (MUSCLE) software (Edgar et al. 2004). The Neighbor-Joining (NJ) phylogenetic trees were constructed using the MEGA 7 software with 1000 bootstrap replicates, and the Sequence Demarcation Tool version 1.2 (SDT v1.2) analyzed the similarity between the sequences (Muhire et al. 2014; Kumar et al. 2016).

Construction of infectious clones

The full-length infectious clones of PEMV-1-YDL and PEMV-2-YDL were constructed using a one-step assembly strategy as shown in Fig. 4. The primers were designed according to the whole genome sequences of the PEMV-1-YDL and PEMV-2-YDL isolates, ensuring an overlap of 21–33 bp nucleotides at the end of the adjacent segments. To obtain full-length infectious clones of PEMV-1-YDL and PEMV-2-YDL, PEMV-1 and PEMV-2 were divided into three fragments for amplification. Primer pairs pCB301-PEMV-1-1F/PEMV-1-1R, PEMV-1-2F/PEMV-1-2R, and PEMV-1-3F/pCB301-PEMV-1-3R (Additional file 1: Table S1) were used to amplify the three adjacent DNA fragments (PEMV-1-1, PEMV-1-2, and PEMV-1-3) of PEMV-1-YDL, respectively. Another three primer pairs, pCB301-PEMV-2-1F/PEMV-2-1R, PEMV-2-2F/PEMV-2-2R, and PEMV-2-3F/pCB301-PEMV-2-3R (Additional file 1: Table S1), were used to amplify DNA fragments PEMV-2-1, PEMV-2-2, and PEMV-2-3 of PEMV-2-YDL, respectively. PCR reactions were performed using PrimeSTAR® GXL DNA Polymerase (TaKaRa Biotechnology, Dalian, China) with cDNAs of the PEMV-1 and PEMV-2 isolates from Dali as the templates. The PCR products were gel-purified and ligated into plant binary vector pCB301-2X35S-MCS-HDRz-tNOS-1 after digestion with the FastDigest restriction enzymes *Sma* I and *Stu* I (TaKaRa Biotechnology, Dalian, China) using the ClonExpress® Ultra One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The resulting recombinant plasmids pCB301-PEMV-1-YDL and pCB301-PEMV-2-YDL with correct sequencing were transformed into *Agrobacterium tumefaciens* EHA105 by the freezing–thawing method (Holsters et al. 1978).

Plant inoculation

The *Agrobacterium tumefaciens* strain EHA105 containing the complete nucleotide sequence of PEMV-1-YDL or PEMV-2-YDL was incubated in Luria–Bertani broth supplemented with Kanamycin and Rifampicin (50 mg/L each) overnight at 28°C. The Agrobacteria cells were collected and resuspended with inoculation buffer (10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone) and grown until the optical density at 600 nm reached 0.8–1.0. The bacterial cells were further cultured at 28°C for 3 h. The two infectious clones were injected individually or as a mixture into leaves of four-week-old *N. benthamiana* and two-week-old pea plants, respectively.

Transmission electron microscopy of the viral particles

Leaf tissues (0.1 g) of the pea plants inoculated with PEMV-1-YDL and PEMV-2-YDL were ground with liquid nitrogen and homogenized in 1 mL of 0.01 mol/L phosphate buffer (pH 7.2). After centrifugation at 11,300 *g* for 15 min, the supernatant was placed on a copper grid for a couple of minutes and then removed using a piece of filter paper. The grid was stained with a drop of 2% tungsten phosphate solution (pH 7.0) and then dried at room

temperature. The grid was then observed by transmission electron microscopy (FEI TECNAL G2 Spirit, USA) in the Yunnan Academy of Agricultural Sciences, Kunming, China, at a voltage of 80 kV and a magnification of 42,000×.

Abbreviations

aa	Amino acid
bp	Base pair
BrYV	Brassica yellows virus
CP	Coat protein
GSP	Gene-specific primer
kDa	Kilodalton
MP	Movement protein
nt	Nucleotide
ORF	Open reading frame
PEMV	Pea enation mosaic virus
PEMV-1	Pea enation mosaic virus 1
PEMV-2	Pea enation mosaic virus 2
PLRV	Potato leaf roll virus
RACE	Rapid amplification of cDNA ends
RdRp	RNA-dependent RNA-polymerase
RNA-3	Smaller RNA
RTP	Read-through protein
RT-PCR	Reverse transcription polymerase chain reaction

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00169-x.

Additional file1: Table S1. Primers used for detection and amplification of PEMV-1 and PEMV-2. **Table S2.** Percent nucleotide (nt) identity among three amplicons (each of 801 bp and 483 bp) of four sampling sites. **Table S3.** Percent nucleotide (nt) and deduced amino acid (aa) identity of ORF3 and ORF5 of PEMV-1-YDL. Comparison of PEMV-1-YDL and known PEMV-1 isolates.

Additional file2: Figure S1. Plant phenotypes and PEMV detection after agro-infiltration of PEMV-1-YDL and PEMV-2-YDL, either singly or in various combinations, on *Nicotiana benthamiana* plants. **Figure S2.** The alignment of CP (**a**) and RTP (**b**) amino acid sequences of PEMV-1-YDL and PEMV-1-AT+.

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Authors' contributions
XC, MX, HC, TW, and FL conceived and designed the experiments. XC, MX, HC, YZ, and KL performed the experiments. PL contributed reagents/materials/analysis tools. XC, MX, and FL wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

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Competing interests
The authors declare that they have no competing interests.

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