Pod pepper vein yellows virus, a new recombinant polerovirus infecting Capsicum frutescens in Yunnan province, China

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Short report

Keywords: pepper vein yellows virus, recombination, Polerovirus, readthrough domain

DOI: https://doi.org/10.21203/rs.3.rs-103456/v2

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Abstract

Pepper vein yellows viruses (PeVYV) are phloem-restricted viruses in the genus Polerovirus, family Luteoviridae. Typical viral symptoms of PeVYV including interveinal yellowing of leaves and upward leaf curling were observed in pod pepper plants (Capsicum frutescens) growing in Wenshan city, Yunnan province, China. The complete genome sequence of a virus from a sample of these plants was determined by next-generation sequencing and RT-PCR. Pod pepper vein yellows virus (PoPeVYV) (MT188667) has a genome of 6015 nucleotides, and the characteristic genome organization of a member of the genus Polerovirus. In the 5’ half of its genome (encoding P0 to P4), PoPeVYV is most similar (93.1% nt identity) to PeVYV-3 (Pepper vein yellows virus 3) (KP326573) but diverges greatly in the 3’-part encoding P5, where it is most similar (91.7% nt identity) to tobacco vein distorting virus (TVDV, EF529624) suggesting a recombinant origin. Recombination analysis predicted a single recombination event affecting nucleotide positions 4126 to 5192 nt, with PeVYV-3 as the major parent but with the region 4126-5192 nt derived from TVDV as the minor parent. A full-length clone of PoPeVYV was constructed and shown to be infectious in C. frutescens by RT-PCR and the presence of icosahedral viral particles.

Background

Pepper vein yellows viruses (PeVYV) are phloem-restricted viruses in the genus Polerovirus, family Luteoviridae and are currently classified into six species (International Committee on Taxonomy of Viruses [ICTV] 2019 release), named Pepper vein yellows virus 1 to 6 [1-6]. They have 86.2-94.6% nucleotide identity between them. Polerovirus genomes have seven open reading frames (ORF0 to ORF5 and ORF3a), putatively encoding proteins P0 to P5 and P3a [6]. Recombination is an important source of genetic variability in viruses, particularly for viruses possessing an RNA genome. PeVYVs have quite close nucleotide similarities to tobacco vein distorting virus (TVDV) in the 5’ half of their genomes, and are considered to have arisen from recombination between TVDV and other poleroviruses [4, 7, 8]. We here report a new recombinant of PeVYV with high identity to PeVYV-3 in the 5’ half of its genome and to TVDV in the 3’ part.

Main Text

Pod pepper (Capsicum frutescens) is widely planted in China, especially around Wenshan city, Yunnan province, and viral diseases have now also become a major threat to pepper production in Yunnan. During July 2019, 89 pepper leaf samples were collected from three different fields in Wenshan. These samples were collected from Da Longshu village (a, 45 samples), Bai Shiyan village (b, 28 samples) and Da Shuduan village (c, 16 samples). All had typical viral symptoms of interveinal leaf yellowing and fruit discoloration (Fig. S1). These leaves (0.4 g per sample) were mixed into three pooled samples by origin, and then sent to the Central Laboratory of Zhejiang Academy of Agricultural Sciences (Hangzhou, China) for Next-generation RNA-Seq sequencing (NGS). A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. RNA integrity was checked by Agilent 2100 Bioanalyzer (Agilent Technologies). The TruSeq RNA Sample Preparation Kit (Illumina) was used to construct cDNA libraries according to the manufacturer’s instructions. An Illumina NovaSeq 6000 platform with PE150 bp and CLC Genomics Workbench 20 (QIAGEN) was used for sequencing and data analysis. A total of 36,430,754 (a: 17,322,690, b: 11,735,694 and c: 7,372,370) paired-end reads were obtained, and 432,848 contigs (a: 143,689, b: 113,012 and c: 176,147) were generated de novo and compared with sequences in the GenBank nt using BLASTn, and 7 contigs were identified with E-values of zero. Contig_62 was 5992 nt long and had high identities (>87.5%) to the genome of PeVYV-3 (Pepper vein yellows virus 3) (KP326573,[3]) and the other contigs were matched with Chilli ringspot virus (ChiRSV, 3 contigs), Chilli veinal mottle virus (ChiVMV, 1 contig), Tomato zonate spot virus (TZSV, 1 contig) and Cucumber mosaic virus (CMV, 1 contig).

In order to verify the virus sequences, total RNA was isolated from each sample using TRIzol™ Reagent (Invitrogen) in compliance with the manufacturer’s instructions. Reverse transcription (RT) polymerase chain reaction (PCR) analyses were performed using the ReverTra Ace qPCR RT Master Mix (Toyobo) and KOD-plus-Neo (Toyobo) following the manufacturer’s protocol. RT was performed at 42°C for 60 min with random primers followed by 72°C for 10 min. The cycling conditions for the subsequent PCR were: 98°C 3min, and then 35 cycles of 98°C for 30s, 55°C for 30s, 68°C for 1kb/min; and 68°C for 10min. Primers designed from Contig_62 were used to amplify a coat protein (CP) fragment of the virus (PeVYV-CP) (Table. S1). Fragments of the expected size (621bp) were obtained in 58 of the 89 symptomatic samples but not from healthy plants (raised from seed in the laboratory). After adding dATP at the 3'-terminal of the PCR products using1 μL Taq™ (TaKaRa) at 72°C for 10min, they were cloned into pGEM-T Easy vector (Promega) and sequenced commercially (Sangon Biotech). The sequences were aligned using MUSCLE, and pairwise nucleotide sequence comparisons were done using the SDT (Species Demarcation Tool) v1.2 program[9, 10]. The 116 amplicons were 93.0-98.4% identical to PeVYV-3, and so we tentatively designated this virus in pod pepper as Pod Pepper vein yellows virus (PoPeVYV).

In order to acquire the full-length sequence of PoPeVYV in pod pepper, 5’ and 3’RACE reactions were then performed to obtain the complete terminal sequences. In brief, the 3’ end of the viral RNA was polyadenylated using Poly(A) Tailing Kit (Invitrogen) and first-strand
cDNA was synthesized using M4T primer (Table. S1). The 3’ terminal end was PCR amplified from the cDNA using M4 and 3’-RACE-F (Tables. S1). Similarly, the 5’ end of the cDNA, after purification by treatment with Gel extraction Kit (Omega) was polyadenylated using the Poly(A) Tailing Kit. The purified cDNA/RNA heterocomplex was ligated with ZHM1 using T4 RNA ligase (Thermo Scientific) and PCR amplified using ZHM2 and 5’ RACE-R (Table. S1; Fig. 1A and B). To avoid errors in sequence assembly, the whole viral sequence was then amplified using two overlapping sections with the primer pairs PoPeVVY-1 and PoPeVVY-2 (Table. S1; Fig. 1C). RT-PCR products (expected sizes 4614 bp and 2824bp) were obtained using the methods of KOD-plus-Neo (Toyobo) and the two products (which overlapped by 1423 bp) were cloned into the pGEM-T Easy Vector (Promega) and sequenced. We obtained 14 amplicons of PoPeVVY-1 and 4 amplicons of PoPeVVY-2, and found that 12 amplicons of PoPeVVY-1 exactly matched the overlapping region of PoPeVVY-2. The complete sequence was 6015 nt long (GenBank accession number: MT188667).

PoPeVVY has a genome organization characteristic of members of the genus Polerovirus, with seven predicted genes encoding proteins P0 to P5 and P3a [6] (Fig. 1A). Over its entire genome our new virus is related (87.8% nucleotide identity) to Tobacco vein distorting virus (TVDV, accession EF529624) and had 85.0% nt identity to the (Chinese) PeVVY-3 (accession KP300822) (Table. 1). However, the 5’ half (nts 1-4251) of PoPeVVY has 93.1% nt identity to the corresponding region of PeVVY-3, and 87.7% nt identity to that of TVDV. The 3’ half (nts 4252-6015) of PoPeVVY has only 64.1% nt identity with PeVVY-3, but 91.7% nt identity with TVDV (Fig. S2). These results indicated that PoPeVVY in pod pepper might be a recombinated virus with PeVVY-3 and TVDV as parents.

Poleroviruses are prone to recombination among themselves or with viruses belonging to other genera and the relationships between PoPeVVY, the previously described PeVVY isolates and TVDV suggests that there has been a recombination event affecting the 3’-end of the genome. This was confirmed using a variety of methods on the RDP4 recombinant platform [11]. A single recombination event affecting nucleotide positions 4126-5952, with TVDV and PeVVY-3 as the respective minor and major parents was consistently identified using GENECONV (P value of 1.035 E-93), RDP (8.410 E-85), BootScan (7.726 E-80), MaxChi (4.294 E-35), Chimera (7.719 E-05), SIScan (3.341 E-68), and Phyldp (2.331 E-15) (Fig. 2). Alignment of amino acid sequences of the PoPeVVY proteins with those of the six species of PeVVYs and TVDV also indicated a recombination event (Table. 1). Phylogenetic trees were constructed from the complete genome sequences, 5’-half or 3’-half of other poleroviruses and an enamovirus as an outgroup (Fig. S3). PoPeVVY, PeVVYs and TVDV formed an independent group separate from the other poleroviruses. The analysis confirmed that the genome and 3’ half of PoPeVVY was similar to TVDV, but that the 5’ half of PoPeVVY was similar to several PeVVYs (Fig. S3). This is reflected in the phylogenetic analysis of the amino acid sequences of the separate gene products [12]: the P0 of PoPeVVY is most similar to PeVVY-1/4, P1/P2/P3/P4 are closely related to other PeVVYs but the RTD is most similar to TVDV (Fig. 3). However, proteins encoded by the 5’ half of the genome (P0 to P4) had the highest identity to those of PeVVY-3 and PeVVY-6, whereas proteins translated from the 3’ half (P5/RTD) were more closely related to those of TVDV (Table. 1, Fig. 3). The various PeVVYs recognized usually have distinct P0 protein sequences and the topology of the P0 phylogenetic tree may depict the ongoing selection for a protein exhibiting better RNA silencing suppression capacity after a recent host jump [4, 7, 13]. The P0 of PoPeVVY has 77.5-87.1% amino acid identity and 85.2-90.5% nucleotide identity to that of the other PeVVYs, and 76.3% amino acid identity (81.3% nucleotide identity) to that of TVDV (Table. 1). In the P0 phylogenetic tree PoPeVVY was most similar to PeVVY-1 and PeVVY-4 and was clearly more similar to the PeVVYs than to TVDV (Fig. 3). These results suggest that PoPeVVY is a new recombinant polerovirus.

An infectious clone of the virus was constructed for further investigation. RT-PCR was performed using KOD-plus-Neo (Toyobo) following the manufacturer’s protocol. To generate infectious clones, the CloneExpress MultiS One Step Cloning Kit (Vazyme) was used for homologous recombination. Two overlapping PCR products were amplified with primers (Inf-PoPeVVY-1/2), and recombined with the linearized binary vector pCB301-MD (a modied version of pCB301 [14]), which includes the double 35S promoter and nopaline synthase terminator (NOS) (Fig. S4)[15, 16]. In this way, the full-length PoPeVVY cDNA was inserted between an upstream 35S promoter and a downstream hepatitis delta virus (HDV) ribozyme and NOS terminator in the binary vector to construct pCB-PoPeVVY. The PoPeVVY cDNA was ligated with 3’-terminal of 35S promoter without any extra nucleotide (Fig. S5)[15, 16]. This clone was transformed into Agrobacterium tumefaciens which was then delivered to C. frutescens plantlets by infiltration. There was mild upward leaf curling 45 days after inoculation (Fig. 4A), and RT-PCR using primers to detect the coat protein gene in the newly-emerged non-inoculated leaves showed that viral RNA was present and had spread systemically in all the inoculated plants (12/12) but not in the controls (Fig. 4B). Virions were puriﬁed from C. frutescens leaves using the method described previously [17]. Isometric particles about 25nm in diameter were observed in the puriﬁed preparation from the inoculated plants (Fig. 4C) but not from the controls. RT-PCR and subsequent sequencing conﬁrmed that the virus in the symptomatic (systemic) leaves had the same sequence as the PoPeVVY cDNA clone inoculated (data not show). These results demonstrate the infectivity of the full-length PoPeVVY to C. frutescens.

In fields of cultivated pod pepper in Wenshan, we had observed severe yellowing and curling symptoms with small leaves (Fig. S1). Those symptoms were more severe than we observed in plants inoculated with the infectious clone of PoPeVVY by Agrobacterium.
tumefaciens which also did not have the obvious vein yellowing usually caused by PeVYVs. Previous studies have shown that the P0 proteins of poleroviruses display variable RNA silencing suppression activity, and induce distinct symptoms [18, 19]. The P0 of PoPeVYV has only 77.5-87.1% amino acid identity to the other PeVYVs, which could account for the distinct symptoms but the field plants were also infected with other viruses (PeVYVs, ChiVMV, ChiRSV, CMV etc) as frequently happens in the field and which has also been reported in Taiwan and Thailand [20, 21]. The severe viral symptoms in the field may therefore be a synergistic effect of mixed infection.

Recombination is an important source of genetic variability in viruses, particularly for viruses possessing an RNA genome. PeVYVs have higher identities to TVDV in the 5’ half of the genome and are considered to be recombinants of TVDV and other poleroviruses [4, 7, 8]. The new recombinant identified here has higher identity to TVDV at the 3’ part of genome, indicating a different sort of recombinant event. The 5’ half of the PoPeVYV genome shares high identity with PeVYV-3, which was reported from Hunan province in China [3]. The 3’ half is much more distant from PeVYV-3 but is more homologous with that of TVDV. TVDV cause a devastating tobacco disease in many tobacco producing areas include Hunan, Guizhou and Yunnan provinces in China [22, 23]. Additionally, PeYVY has been reported to infect tobacco in Guizhou province suggesting that co-infection of tobacco might have provided an opportunity for recombination [24]. Recombination poses a problem for classification. The currently-recommended species demarcation criteria in the family Luteoviridae suggest that different species should have >10% difference in amino acid sequence identity in any gene product from their closest relative. The P0 and P5 proteins of PoPeVYV have respectively 12.9-23.7% and 10.3-70.5% difference in amino acid identity to those of PeVYV1-6 (Table. 1). If applied here, those criteria suggest that PoPeVYV could be representative of a distinct species.

Conclusions

A full-length sequence of Pod pepper vein yellows virus (MT188667) was determined. Alignment and recombination analysis predicted a single recombination event with PeVYV-3 as the major parent but with the region 4126-5192 nt derived from TVDV as the minor parent. PoPeVYV is a new recombinant polerovirus infecting C. frutescens in Yunnan province, China.

Abbreviations

nt: Nucleotide; RACE: Rapid amplification of cDNA ends; RT-PCR: Reverse transcription polymerase chain reaction; PoPeVYV: Pod Pepper vein yellows virus; PeVYV: Pepper vein yellows virus; TVDV: Tobacco vein distorting virus

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The complete genome sequences of PoPeVYV were submitted to the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the accession number is MT188667.

Competing interests

The authors declare that they have no conflict of interest.

Funding

This work was financially supported by Ningbo city Natural Science Foundation of China (2019A610403), Chinese Agriculture Research System (CARS-24-C-04). This work also was supported by grant-in-aid from State Key Laboratory for Managing Biotic and Chemical Threats to the Quality and Safety of Agroproducts (KF20190107) and K. C.Wong education foundation.

Authors’ contributions
FY, JP conceived and designed the experiments. SW, EY, LL collected the samples. XM, KZ, YY, LL and MH performed the experiments. HC, YL and JC analyzed the data. KZ, YY, JP and FY wrote the paper. All authors read and approved the final manuscript.

**Acknowledgements**

We thank Professor Mike Adams for manuscript correction.

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**References**

1. Dombrovsky A, Glanz E, Lachman O, et al: The complete genomic sequence of pepper yellow leaf curl virus (PYLCV) and its implications for our understanding of evolution dynamics in the genus polerovirus. PLoS One 2013, 8:e70722.

2. Knierim D, Tsai WS, Kenyon L: Analysis of sequences from field samples reveals the presence of the recently described pepper vein yellows virus (genus Polerovirus) in six additional countries. Arch Virol 2013, 158:1337-1341.

3. Liu M, Liu X, Li X, et al: Complete genome sequence of a Chinese isolate of pepper vein yellows virus and evolutionary analysis based on the CP, MP and RdRp coding regions. Arch Virol 2016, 161:677-683.

4. Lotos L, Olmos A, Orfanidou C, et al: Insights Into the Etiology of Polerovirus-Induced Pepper Yellows Disease. Phytopathology 2017, 107:1567-1576.

5. Maina S, Edwards OR, Jones RA: First Complete Genome Sequence of Pepper vein yellows virus from Australia. Genome Announc 2016, 4.

6. Murakami R, Nakashima N, Hinomoto N, et al: The genome sequence of pepper vein yellows virus (family Luteoviridae, genus Polerovirus). Archives of Virology 2011, 156:921-923.

7. Ghosh S, Kanakala S, Lebedev G, et al: Transmission of a New Polerovirus Infecting Pepper by the Whitefly Bemisia tabaci. J Virol 2019, 93.

8. Kamran A, Lotos L, Amer MA, et al: Characterization of Pepper leafroll chlorosis virus, a New Polerovirus Causing Yellowing Disease of Bell Pepper in Saudi Arabia. Plant Disease 2018, 102:318-326.

9. Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004, 32:1792-1797.

10. Muhire BM, Varsani A, Martin DP: SDT: a virus classification tool based on pairwise sequence alignment and identity calculation. PLoS One 2014, 9:e108277.

11. Martin DP, Murrell B, Golden M, et al: RDP4: Detection and analysis of recombination patterns in virus genomes. Virus Evol 2015, 1:ev003.

12. Kumar S, Stecher G, Li M, et al: MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol Biol Evol 2018, 35:1547-1549.

13. Koeda S, Homma K, Kamitani M, et al: Pepper vein yellows virus 9: a novel polerovirus isolated from chili pepper in Indonesia. Arch Virol 2020, 165:3017-3021.

14. Xiang C, Han P, Lutziger I, et al: A mini binary vector series for plant transformation. Plant Mol Biol 1999, 40:711-717.

15. Shi BJ, Ding SW, Symons RH: Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus. J Gen Virol 1997, 78 ( Pt 5):1181-1185.

16. Scholthof HB: Rapid delivery of foreign genes into plants by direct rub-inoculation with intact plasmid DNA of a tomato bushy stunt virus gene vector. J Virol 1999, 73:7823-7829.

17. Mo X-H, Chen Z-B, Chen J-P: Molecular identification and phylogenetic analysis of a viral RNA associated with the Chinese tobacco bushy top disease complex. Annals of Applied Biology 2011, 158:188-193.

18. Kozlowska-Makulska A, Guilley H, Szynel MS, et al: P0 proteins of European beet-infecting poleroviruses display variable RNA silencing suppression activity. J Gen Virol 2010, 91:1082-1091.
19. Almasi R, Miller WA, Ziegler-Graff V: Mild and severe cereal yellow dwarf viruses differ in silencing suppressor efficiency of the P0 protein. Virus Res 2015, 208:199-206.

20. Laprom A, Nilthong S, Chukeatirote E: Incidence of viruses infecting pepper in Thailand, Biomol Concepts 2019, 10:184-193.

21. Cheng YH, Deng TC, Chen CC, et al: First Report of Pepper mottle virus in Bell Pepper in Taiwan, Plant Dis 2011, 95:617.

22. Mo X-H, Qin X-Y, Tan Z-X, et al: First Report of Tobacco Bushy Top Disease in China, Plant Disease 2002, 86:74-74.

23. Liu F, Tan G, Li X, et al: Simultaneous detection of four causal agents of tobacco bushy top disease by a multiplex one-step RT-PCR. Journal of Virological Methods 2014, 205:99-103.

24. Wang LS, He QC, Chen XJ, et al: First Report of Pepper vein yellows virus Infecting Tobacco (Nicotiana tabacum) Naturally in China. Plant Disease 2017, 101:1556.

Tables

Table 1: Identity percentages between the genomes of PoPeVYV and with those of closely related species

|       | 5'NCR | P0   | P1   | P2   | iNCR | P3a  | P3   | P4   | RTD | 3'NCR | Full genome |
|-------|-------|------|------|------|------|------|------|------|-----|-------|-------------|
| nt    | nt    | nt   | nt   | nt   | nt   | nt   | nt   | nt   | nt  | nt    | nt          |
| PoPeVYV | 100   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100 | 100   | 100         |
| PeVV-1 | 98.0  | 83.5 | 88.9 | 89.8 | 91.9 | 93.4 | 93.3 | 96.3 | 97.8| 97.8   | 94.2        |
| PeVV-2 | 98.0  | 83.9 | 88.9 | 89.9 | 92.1 | 94.7 | 93.8 | 92.5 | 91.1| 92.0   | 93.1        |
| PeVV-3 | 98.0  | 87.1 | 90.5 | 90.8 | 92.6 | 93.9 | 92.7 | 95.1 | 100 | 97.8   | 94.2        |
| PeVV-4 | 66.0  | 85.1 | 89.2 | 90.1 | 91.5 | 94.4 | 93.7 | 92.5 | 95.6| 94.9   | 92.7        |
| PeVV-5 | 96.0  | 77.5 | 85.2 | 87.2 | 90.3 | 95.0 | 93.5 | 92.5 | 93.3| 94.9   | 94.2        |
| PeVV-6 | 96.0  | 85.1 | 89.6 | 90.5 | 92.9 | 95.0 | 94.1 | 90.0 | 95.6| 94.2   | 94.7        |
| TVDV   | 81.6  | 76.3 | 81.3 | 77.2 | 83.0 | 90.8 | 88.6 | 84.8 | 95.6| 87.7   | 89.8        |
|       |       |      |      |      |      |      |      |      |      |       |             |

R = noncoding region, iNCR = intergenic NCR, aa = amino acid, and nt = nucleotide. Highest percentages are underlined and in bold.

PoPeVYV (Pepper vein yellows virus, MT188667)