Characterization of Pepper leafroll chlorosis virus, a New Polerovirus Causing Yellowing Disease of Bell Pepper in Saudi Arabia

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Abstract

During the growing seasons of 2014 through 2016, a total of 336 leaf samples from bell pepper (showing leafroll and interveinal yellowing) and arable weeds were collected from Riyadh region, Saudi Arabia. The use of a polerovirus generic reverse transcription (RT)-PCR assay confirmed their presence in the bell pepper samples. Sequencing of the generic amplicon revealed high similarity (87.6 to 98.1% in nt) with four poleroviruses: *Tobacco vein distorting virus*, *Pepper vein yellows virus*, *Pepper yellows virus*, and *Pepper yellow leaf curl virus*. To further characterize one of these isolates (105D), a larger part of the genome (~1,300 nt) spanning approximately from the 3' end of ORF2 to the middle of ORF3, was amplified and sequenced. Blasting the resulting sequence revealed the low amino acid and nucleotide identity percentages in

the coat protein and movement protein partial genes with viruses deposited in GenBank. Next-generation sequence was used to acquire a larger part of the genome, which resulted in the reconstruction of isolate 105D's partial genome (5,496 nt). Sequence similarity analysis revealed the presence of a divergent polerovirus isolate belonging to a new species that was tentatively named Pepper leafroll chlorosis virus (PeLRCV). Using a specific RT-PCR assay for this isolate confirmed the presence of this new viral species in the symptomatic peppers. Aphid transmission experiments showed that PeLRCV is vectored by *Aphis gossypii* and that it can infect at least five out of the 15 different plants species tested. Based on our findings, PeLRCV is a new member of genus *Polerovirus* in the family *Luteoviridae*.

Bell pepper (*Capsicum annuum* L.) is an economically important crop plant of the nightshade family (Solanaceae). Bell peppers play a major role in Saudi Arabian agriculture. Viral diseases pose a major threat to pepper production, as at least 68 different viruses have been reported to infect pepper plants, resulting in a global yield reduction (Pernezny et al. 2003). Many plant viruses of the genus *Polerovirus* (family *Luteoviridae*), such as *Pepper vein yellows virus* (PeVYV) and *Pepper yellow leaf curl virus* (PYLCV) (Note: This virus name formally refers to a pepper-infecting begomovirus, but it was used for a pepper-infecting polerovirus as well [Dombrovsky et al. 2013]; in this study, the complete name and the acronym PYLCV will refer to the polerovirus only), *Pepper yellows virus* (PeYV) (Lotos et al. 2017), *Beet western yellows virus* (BWYV) (Buzkan et al. 2013), and *Capsicum yellows virus* (CYV) (Gunn and Pares 1990) have been reported to infect pepper. Recent studies have related the infection of pepper by PeVYV, PYLCV, and PeYV with symptoms of interveinal yellowing, stunting, and leaf rolling (pepper yellows disease – PYD) in different countries globally such as Australia, China, Greece, India, Indonesia, Italy, Japan, Mali, the Philippines, Sudan, Taiwan, Thailand, Tunisia, Turkey, and the U.S.A. (Alabi et al. 2015; Alfaro-Fernández et al. 2014; Buzkan et al. 2013; Dombrovsky et al. 2010; Knierim et al. 2013, 2015; Lotos

et al. 2017; Maina et al. 2016; Tan et al. 2015; Tomassoli et al. 2016; Yonaha et al. 1995).

Poleroviruses are a genetically diverse group of viruses that share several distinct characteristics. These include: transmission by aphids in a persistent circulative and nonpropagative manner that involves sophisticated interactions between virus and the aphid vector, being phloem-limited, lack of mechanical transmission, a high degree of vector specificity, and close serological relationships between most members of the genus (Dietzgen et al. 2016; Hogenhout et al. 2008; Katis et al. 2007; Liu et al. 2006; Martin et al. 1990). Virions of poleroviruses are isometric, 25 to 30 nm in size, housing a single-stranded (ss) positive sense RNA molecule of approximately 5.6 to 5.9 kb (Mayo and Ziegler-Graff 1996), with a small genome-linked protein covalently bonded to the 5' end of the genomic RNA (van der Wilk et al. 1997).

During a survey conducted in 2014 in greenhouses of the Riyadh region, bell pepper plants showing symptoms of PYD similar to those induced by the infection of pepper poleroviruses were observed. To our knowledge, there are no reports of members of the genus *Polerovirus* infecting bell pepper in Saudi Arabia. Therefore, the aim of this study was to identify and characterize the causal agent of PYD in Saudi Arabia, which led to the identification of a new polerovirus, tentatively named as Pepper leafroll chlorosis virus (PeLRCV), associated with this disease.

Materials and Methods

Plant sample collection. During the spring seasons of 2014 to 2016, a total of 220 bell pepper samples and 116 from different arable weeds were collected from different locations (Al-Aflaj, Al-Huta, Sajir, Shaqra, and Wadi Nisah) in the Riyadh region, Saudi Arabia. All the collected pepper samples were from symptomatic plants showing PYD symptoms (Fig. 1), whereas the arable weeds were collected irrespectively of the presence of symptoms. Ten weed species were collected, including eight species of local flora (*Amaranthus*

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viridis, *Chenopodium murale*, *Calotropis procera*, *Lactuca saligna*, *L. serriola*, *Malva parviflora*, *Portulaca oleracea*, *Solanum nigrum* and two volunteer species (*Momordica charantia* and *Vigna radiata*).

Screening of plant samples for viruses. Total RNA was extracted from 95 bell pepper and 57 weed samples (selected samples were representatives of different locations visited during the 3-year survey) using a total RNA isolation kit (Jena Bioscience, Germany) according to the manufacturer's protocol. For the identification of poleroviruses, the samples were screened using a degenerate primer pair that amplifies a 593-nt part of the RdRp gene (ORF2) (Lotos et al. 2014). Samples found positive for the presence of poleroviruses with the generic assay were also screened for BWYV (Lotos et al. 2014) and for PYLCV (Dombrovsky et al. 2010) by hyphenation RT-PCR.

For the specific detection of PeLRCV, an RT-PCR was developed using the primer pair PeLRCV5-Up and PeLRCV5-Down (Table 1) which amplifies a 693-nt part of ORF5. Primers were designed based on the sequence retrieved from the next-generation sequencing (NGS) of isolate 105D (LT220496).

To obtain isolate 105D's part of the genome spanning from the 3' end of the RdRp gene to the middle of the coat protein (CP) gene, the nested RT-PCR assay developed by Lotos et al. (2016) was used (Table 1, Fig. 2).

All RT-PCRs were performed in a Mastercycler Nexus Gradient (Eppendorf, Germany) thermal cycler using Takara One Step RT-PCR kit (Takara Bio Inc., Japan). The reaction was set up according to the manufacturer's recommendations. Products were visualized in an 1% agarose gel stained with ethidium bromide, using a 1-kb and 100-bp molecular weight marker (Genedirex). PCR products were directly sequenced in both directions at Advanced Genetic Technology Center (U.S.A.).

Next-generation sequencing of isolate 105D. Small interfering RNAs (siRNAs) were purified from freeze-dried leaf and petiole pepper tissue using the mirPremier microRNA Isolation Kit (Sigma-Aldrich, St. Louis, U.S.A.). Library preparation and sequencing on a MiSeq platform (Illumina Genome Analyzer) were carried out by Life Sequencing (Valencia, Spain). De novo assembly was performed using the Velvet plugin implemented in Geneious software (Biomatters Ltd., Auckland, New Zealand) and the resulting contigs were blasted (BLASTn, BLASTx) against GenBank databases. De novo contigs that exhibited similarity with known poleroviruses were extended using iterative mapping in Geneious.

Similarity and phylogenetic analysis. Identity percentages between Saudi isolates and other poleroviruses was calculated using Geneious (alignment distances). Maximum likelihood phylogenetic trees were constructed using PhyML version 3.1 (Guindon and Gascuel 2003), using the evolutionary model provided by ProtTest version 3.4 (Abascal et al. 2005). Recombination events were predicted using RDP4 (Martin et al. 2010). Alignments were performed with MAFFT version 7 (Katoh and Standley 2013).

Aphid transmission and host range experiments for PeLRCV.

Host range experiments were conducted as described by Yonaha et al. (1995) and Dombrovsky et al. (2010) using a clone of *Aphis gossypii* collected from *Stenolobium stans* and maintained onto *Citrullus lanatus*. Selected samples were tested by specific primers using RT-PCR targeting PYLCV, PeVYV, BWYV, and PeLRCV. One pepper plant (*C. annuum* 'Sonar,' isolate 105D collected from Wadi Nisah) showing leaf rolling and interveinal yellowing symptoms (Fig. 1), singly infected by PeLRCV, served as the initial virus source in this study. Apterae *A. gossypii* were fed on the virus source plant and transferred to healthy pepper plants to multiply the virus source in greenhouse. The acquisition access period (AAP) and inoculation access period (IAP) were 72 and 24 h, respectively. Aphids were transferred in groups of 10 to 15 per test plant. Twelve plants at the three-leaf stage of each of the following species, *Amaranthus viridis*, *C. annuum*, *Cucumis melo*, *Cucurbita pepo*, *Chenopodium amaranticolor*, *C. lanatus*, *Datura stramonium*, *Lagenaria siceraria*, *S. nigrum*, *Solanum lycopersicum*, *Solanum melongena*, *Nicotiana tabacum*, *Petunia hybrida*, *Vicia faba*, and *Vigna radiata* were inoculated. After IAP, plants were sprayed with an insecticide and were kept in insect-proof cages in a greenhouse for symptom development. Symptoms were recorded 30 days post inoculation and all inoculated plants were tested for PeLRCV by RT-PCR with primers developed herein.

Results

RT-PCR assay for generic detection of genus polerovirus.

From the pepper samples screened, 91% (86/95) were found positive for poleroviruses using the generic RT-PCR. The obtained 593-bp product from seven Saudi isolates representing different locations (Al-Aflaj, Al-Huta, Sajir, Shaqra, and Wadi Nisah) was sequenced (105D, Pol-26-SA, Pol-31-SA, Pol-38-SA, Pol-67-SA, Pol-86-SA, and Pol-97-SA, accession nos. LT220496, KY401684, KY401690, KY401685, KY401687, KY401688, and KY401689, respectively)

Fig. 1. Polerovirus-like symptoms on bell pepper plants collected from greenhouses during different field surveys. Symptomatic plants exhibited upward leafroll and interveinal yellowing of leaves (A and B) as well as malformed fruits (C), in contrast to healthy ones (D).

Table 1. Oligonucleotide primers and RT-PCR cycling used to detection of poleroviruses infecting pepper

Target virus(es)/coding regions	Primer pair	Sequence 5' to 3'	Amplicon size (bp)	Thermocycling conditions		References
				RT	PCR	
Polero generic	PolGenUp2	gatgarggtcgytaccg	593	45°C 1 h	94°C 15 min	Lotos et al. 2014
	PolGenDown2	acctcgactttraarc		70°C 15 min	94°C 30 s 58°C 15 s 54°C 10 s 52°C 10 s 72°C 30 s 72°C 5 min	
Pepper yellow leaf curl virus (PYLCV)	F1-PYLCV	ggaggaaggtcragcaacagc	650	45°C 1 h	94°C 15 min	Dombrovsky et al. 2010
	R2-PYLCV	ctatttggggtgtgyarttgac		70°C 15 min	94°C 30 min 60°C 30 s 72°C 30 s 72°C 10 min	
<i>Beet western yellows virus</i> (BWYV)	BWYVUp	accctgctygcgcagc	168	45°C 1 min	94°C 15 min	Lotos et al. 2014
	BWYVDo	gtatttcccyaggtcggctactag		70°C 15'	94°C 30 s 60°C 20 s 72°C 20 s 72°C 5 min	
RdRp to CP	PolGenP5Down	gbcgctacatttngg	1,300	45°C 1 h	94°C 15 min	Lotos et al. 2016
	PolGenCpDown	gatyttataytcagtgaggcyttgag		70°C 15 min	94°C 30 s	
	PolGenUp2	gatgarggtcgytaccg			60°C 10 s	
	PolGenUpNest	gaytgctchggttygaytgg			56°C 10 s 52°C 10 s 72°C 10 s 72°C 5 min	
Pepper leafroll chlorosis virus (PeLRCV)	PeLRCV5-Up	gataattgcttagctgagtctctag	693	45°C 1 h	94°C 15 min	This study
	PeLRCV5-Down	gaaactaaccgcatggtctc		70°C 15 min	94°C 30 s 60°C 30 s 72°C 30 s 72°C 10 min

Fig. 2. Partial genome of Pepper leafroll chlorosis virus (PeLRCV) isolate 105D showing the changes in similarity which indicate possible recombination events. The dashed line shows the position of the ~1,300-nt part spanning from the RdRp-CP used in the initial characterization. The results of the recombination analysis are plotted below the genome. #: Pepper yellows disease group-like, !: PeLRCV unique, *: *Luffa aphid-born yellows virus*-like, &: *Pepper vein yellows virus*/*Potato leafroll virus*-like.

and homology search with BLASTn confirmed the relation of these isolates with members of the genus. None of the samples was positive for PYLCV or BWYV. Furthermore, all the arable weed plant samples were negative for the presence of poleroviruses. (Fig. 3).

Similarity analysis of the generic amplicon showed that the nucleotide (nt) sequence identity among the six Saudi isolates ranged from 96.6 to 98.3% with the amino acid (aa) identity being 94.9 to 100%. Moreover, this region of the RdRp revealed high sequence identity (87.6 to 98.1% and 91.5 to 100% in nt and aa, respectively) of the Saudi Arabian isolates and the isolates deposited in GenBank of the three closely related poleroviruses (PeVYV, PeYV, and PYLCV) belonging in the PYD group (data not shown).

Characterization of isolate 105-D. Isolate 105D, which was among the pepper polerovirus isolates sequenced, was selected for further characterization. Sequencing of the overlapping PCR products (polerovirus generic and RdRp-CP) resulted in one consensus sequence of 1,290 nt representing 20 to 23% of the predicted genome of isolate 105D. This product included the region encoding the C-terminal part of the RdRp, and approximately half of the CP and the movement protein (MP) (Fig. 2). Although isolate 105D exhibited high sequence identity with the PYD group viruses in the RdRp region, it differed significantly with them after the intergenic noncoding region (iNCR), especially on the N-terminal part of CP (Table 2).

Given the differences present in the CP/MP region of the genome, isolate 105D was selected for NGS. The run produced a total of 3,842,585 reads, 875,499 of which corresponded to the 21 to 24 nt siRNAs (22.8%). De novo assembly (*k*-mer 15, N50 = 74) produced 654 contigs, but only 188 were related to plant virus sequences. From

these, 143 were similar to *Bell pepper endornavirus* (BPEV, family *Endornaviridae*, genus *Endornavirus*) and the rest were related to species of the genus *Polerovirus*, mainly with the pepper-infecting poleroviruses (PeVYV and PYLCV) (40 out of the remaining 45 contigs). Iterative mapping of the reads to the polerovirus-related contigs resulted into the reconstruction of a partial polerovirus genome with some minor gaps. These gaps were filled with sanger sequencing, which produced a 5,496-nt partial genome. A total of 11,506 reads from both polarities were mapped (56.6% positive, 43.4% negative) to this partial genome, with coverage ranging from 0 to 498 with a mean of 45.5. The majority of the reads were of the 21 to 22 nt class. De novo contigs corresponding to the 3' end of ORF5 and the 3' NCR were not found, and it was not possible to extend the genomic sequence toward that direction with iterative mapping.

The phylogenetic trees constructed from the aa of the RdRp (Fig. 4) and the CP (Fig. 5) showed the close relationship of isolate 105D with members of the genus polerovirus and more specifically with the pepper-infecting poleroviruses (PYD group). However, the partial genome obtained from isolate 105D exhibited differences in sequence identity with the other virus species of PYD group depending on the part of the genome examined. ORFs 0 to 2 and ORF3a are highly similar to PYLCV-PeVYV in nt and aa, whereas the other three ORFs are less than 75% identical in nt and aa with those viruses. Moreover, the N-terminal part of the CP that corresponds to the first 177 nt was not homologous to any sequence in the database. PeLRCV is least similar to the other pepper poleroviruses in ORF5, with identity scores below 35%. This is because the N-terminal part of P5 lacks the CABYV-like recombination,

Fig. 3. Agarose gel (1%) electrophoresis showing the results of the generic RT-PCR assay (593 bp) for the detection of viruses of the genus *Polerovirus* in selected samples from bell pepper (lanes 1, 2, 3, 5, 6, 7, 11, 12) and other selected weeds: *Amaranthus viridis*, *Chenopodium murale*, *Calotropis procera*, *Lactuca saligna*, *L. serriola*, and *Vigna radiata* (lanes 4, 8, 9, 10, 13, 14) collected from Riyadh region, Saudi Arabia. No RT-PCR amplification was observed from uninfected pepper sample (lane 15). Lane 1: Gene Direx 1 Kb DNA ladder.

Table 2. Similarities (%) between isolate 105D and *Tobacco vein distorting virus* (TVDV) (EF529624), *Pepper vein yellows virus* (PeVYV) (AB594828), *Pepper yellows virus* (PeYV) (LT559484), and *Pepper yellow leaf curl virus* (PYLCV) (HM439608)

ORFs	Length (nt)	Position (nt start–nt end)	TVDV ^a	PeVYV ^a	PeYV ^a	PYLCV ^a
ORF0	750	50–799	77.9 / 82.7*	84.3 / 89.1	92.0 / 94.5	92.8 / 94.8
ORF1	1,965	174–2,138	78.0 / 83.8	91.4 / 92.5	96.0 / 95.0	96.2 / 95.3
ORF2 (RdRp) ^b	1,773	1,658–3,430	90.2 / 89.2	94.9 / 94.0	97.6 / 96.6	96.4 / 94.9
ORF3a	138	3,512–3,649	93.3 / 87.0	95.6 / 87.7	97.8 / 93.5	77.8 / 93.3
ORF3 (CP)	615	3,630–4,244	73.0 / 72.0	73.5 / 73.1	74.4 / 73.6	73.5 / 72.6
ORF4 (MP)	462	3,664–4,125	55.7 / 72.8	57.6 / 73.4	55.7 / 73.6	55.7 / 72.4
ORF5 ^c	1,252	4,245–5,496	13.5 / 26.5	29.1 / 34.7	27.6 / 35.7	18.2 / 29.4

^a Bold: difference in amino acid (aa); italic: differences in nucleotides (nt).

^b After the frameshift.

^c After the readthrough, partial ORF.

present in the other pepper polioviruses, which is replaced by a *Luffa aphid-born yellows virus* (LABYV)-like one (Fig. 2). Moreover, the recombination is followed by a C-terminal part which is moderately identical to PeVYV and *Potato leafroll virus* (PLRV) (55 to 60% in aa). This recombination event was supported by six out of the seven algorithms in RDP4 (RDP, GENECONV, BootScan, MaxChi, Chimera, and 3seq) (Boni et al. 2007; Martin et al. 2005; Padidam et al. 1999; Posada and Crandall 2001; Smith 1992).

The 5,496-nt long partial genome sequence was deposited in European Nucleotide Archives under the accession number LT220496, as a new polerovirus tentatively named as Pepper leafroll chlorosis virus (PeLRCV).

PeLRCV specific RT-PCR assay. Pepper samples positive for poleroviruses were tested for the presence of PeLRCV using the specific detection RT-PCR developed herein. The PeLRCV specific amplicon of 693 bp was amplified from all isolates tested (Fig. 6) and was sequenced from the six isolates identified as members of the PYD group (the same ones that were sequencing of the generic amplicon) (PeLRCV-26-SA-ORF5, PeLRCV-31-SA-ORF5, PeLRCV-38-SA-ORF5, PeLRCV-67-SA-ORF5, PeLRCV-86-SA-ORF5, and PeLRCV-97-SA-ORF5, accession nos. KY401678, KY401679, KY401680, KY401681, KY401682, and KY401683, respectively). The percentage nt identity ranged from 98.4 to 100% between them and 105D, indicating that all of them are isolates of the same virus species.

Host range studies and insect transmission of PeLRCV. From the 15 plant species used in the aphid transmission experiments, symptoms developed in only four: *C. annuum*, *C. melo*, *D. stramonium*,

and *S. nigrum*. Symptoms included interveinal yellowing, leaf rolling, vein yellowing, and small leaves (Table 3, Fig. 7). All inoculated plants were tested with the PeLRCV-specific RT-PCR assay and the results confirmed the viral infection in the aforementioned four plant species as well as in *C. amaranticolor* that remained asymptomatic (Table 3, Fig. 8).

Discussion

The present survey of bell pepper crops, conducted between 2014 and 2016 in Riyadh region, Saudi Arabia, identified the presence of a new virus associated with PYD. The incidence of polerovirus infection on bell pepper was high (91%), and although a substantial number of symptomatic plants were present in every location sampled, aphids (only *A. gossypii*) were rarely observed on infected plants.

Sequencing of the obtained generic amplicons revealed the high percentage identities in nucleotides and amino acids of all Saudi isolates with the three closely related pepper poleroviruses (PeVYV, PeYV, and PYLCV) and *Tobacco vein distorting virus* (TVDV) that suggest the phylogenetic relationship with these viruses. These viral species share a common ancestry and have a highly identical RdRp region, which cannot be used for classification. However, even small differences in the aa sequence of the generic amplicon can indicate the presence of a divergent viral isolate (Lotos et al. 2014).

For this reason, isolate 105D was selected for further characterization starting with the region spanning from the 3'-end of the RdRp until the middle of the CP, which revealed the substantial differences between the Saudi isolate and the already characterized

Fig. 4. Maximum likelihood phylogenetic tree constructed using the aa sequence of the RdRp after the overlap. SH-aLRT scores above 80 are given above each node and the bar on the left indicate substitutions/site. Abbreviations and accession numbers of the isolates used are: BChV (*Beet chlorosis virus*, AF352024), BMV (*Beet mild yellowing virus*, DQ132996), BrYV (*brassica yellows virus*, KF015269), BWYV (*Beet western yellows virus*, AF473561), CABYV (*Cucurbit aphid-borne yellows virus*, JQ700305), CLRDV (*Cotton leafroll dwarf virus*, NC_014545), CpCSV (*Chickpea chlorotic stunt virus*, AY956384), CtRLV (*Carrot red leaf virus*, AY695933), CYDV-RPS (*Cereal yellow dwarf virus-RPS*, AF235168), CYDV-RPV (*Cereal yellow dwarf virus-RPV*, EF521827), MABYV (*Melon aphid-borne yellows virus*, JQ700307), MYDV-RMV (*Maize yellow dwarf virus-RMV*, NC_021484), PEMV-1 (*Pea enation mosaic virus 1*, NC_003629), PeVYV (*Pepper vein yellows virus*, AB594828), PeYV (*pepper yellows virus*, LT559484) PLRV (*Potato leafroll virus*, AF453390), PMCV (*pea mild chlorosis virus*, JF507725), PYLCV (*pepper yellow leaf curl virus*, HM439608), SAbYV (*Suakwa aphid-borne yellows virus*, JQ700308), ScYLV (*Sugarcane yellow leaf virus*, JF925154), TuYV (*Turnip yellows virus*, X13063), TVDV (*Tobacco vein distorting virus*, EF529624), and WYDV (*wheat yellow dwarf virus*, FM865413).

poleroviruses. These differences indicated that isolate 105D is either a highly divergent isolate or a new virus, so an NGS analysis followed to acquire the complete genome. The 5,496 nt long genome that was reconstructed from the NGS had the typical organization of

poleroviruses. However, it was not possible to obtain the ~500 nt of the 3'-end of the genome, which include the latter half of ORF5 and the 3' NCR, even when the PYD group poleroviruses were used as reference genomes, indicating that either the siRNAs for this part

Fig. 5. Maximum likelihood phylogenetic tree constructed using the aa sequence of the coat protein. SH-aLRT scores above 80 are given above each node and the bar on the left indicate substitutions/site. Abbreviations and accession numbers of the isolates are the same as in Figure 4.

Fig. 6. Agarose gel (1%) showing the results of the Pepper leafroll chlorosis virus-specific RT-PCR detection assay of the selected samples collected from different locations in Riyadh region (lanes 1 to 14). The arrow indicates the expected size of 693 bp. Lane M: Gene Direx 100 bp DNA ladder (pictured on the left). No RT-PCR amplified product was observed from uninfected bell pepper (lane 15).

were absent, or this part has a completely new sequence that is not homologous to any virus.

Similar to the other pepper-infecting poleroviruses, the genome of isolate 105D has a TVDV-like 5' half, which is almost identical to PYLCV (Table 2). However, after ORF3a the similarity shifts and the 3' half of the genome is unique among pepper poleroviruses. The N-terminal part of the CP does not appear to be homologous to any sequence, and the rest of the sequence has low identities to PYLCV (85.5% in aa), MP is highly divergent (55 to 58% in aa), and the N-terminal of P5 has a LABYV-like recombination in contrast to the CABYV one present in the other viruses of the group (Table 2, Fig. 2). The phylogenetic trees constructed show that both halves of the genome share a common phylogenetic origin with the rest of the pepper poleroviruses (Figs. 4 and 5), which is further

supported by the high identities of the 5' half. However, the genome of 105D appears to be heavily shaped by recombination events. The nonhomologous N-terminal part of the CP, the LABYV-like part of the P5, and the unidentified end of ORF5 indicate that multiple recombination events possibly occurred during this isolate's emergence. Due to the highly divergent genome of isolate 105D and the fact that three ORFs (3, 4, and 5) are below the 10% limit set by the International Committee on Taxonomy of Viruses for demarcation (D'Arcy and Domier 2005), we propose the classification of isolate 105D in a new species of the genus *Polerovirus* tentatively named Pepper leafroll chlorosis virus (PeLRCV).

Since the pepper-infecting poleroviruses exhibit high sequence identity in the 5' part of their genome and PeLRCV has a unique ORF5 sequence, this region was selected for the design of primers

Table 3. Host range and description of symptoms induced by Pepper leafroll chlorosis virus using *A. gossypii* as vector

Plant species	Types of symptoms expression	Number of symptomatic plants ^a	RT-PCR results ^b
<i>Capsicum annuum</i>	Vein yellowing, leafrolling	12/12	+
<i>Datura stramonium</i>	Intervenal chlorotic patches	10/12	+
<i>Petunia hybrida</i>	Asymptomatic	0/12	–
<i>Vicia faba</i>	Asymptomatic	0/12	–
<i>Cucumis melo</i>	Mosaic, small leaves	11/12	+
<i>Chenopodium amaranticolor</i>	Asymptomatic	9/12	+
<i>Solanum nigrum</i>	Intervenal mosaic	10/12	+
<i>Amaranthus viridis</i>	Asymptomatic	0/12	–
<i>Cucurbita pepo</i>	Asymptomatic	0/12	–
<i>Citrullus lanatus</i>	Asymptomatic	0/12	–
<i>Lagenaria siceraria</i>	Asymptomatic	0/12	–
<i>Solanum lycopersicum</i>	Asymptomatic	0/12	–
<i>Solanum melongena</i>	Asymptomatic	0/12	–
<i>Nicotiana tabacum</i>	Asymptomatic	0/12	–
<i>Vigna radiata</i>	Asymptomatic	0/12	–

^a Number of symptomatic plants after inoculation/total number of plants.

^b Reverse transcription polymerase chain reaction detection assay in host range plant species. +: positive, –: negative.

Fig. 7. Different symptom expressions of plant species included in host range of Pepper leafroll chlorosis virus: **A**, *Capsicum annuum*: control. **B**, *C. annuum*: inoculated; vein yellowing and leaf rolling. **C**, *Cucumis melo*: inoculated; mosaic. **D**, *Datura stramonium*: inoculated; interveinal chlorotic patches. **E**, *Solanum nigrum*: inoculated; interveinal mosaic. **F**, *Chenopodium amaranticolor*: inoculated; asymptomatic.

for the specific detection of PeLRCV. Using this specific assay, PeLRCV was found to infect all the bell pepper samples that were positive for poleroviruses, and negative for PYLCV and BWYV, and sequencing of this amplicon from six samples confirmed the presence of PeLRCV. Moreover, according to NGS, no other known virus was found in sample 105D apart from BPEV and PeLRCV, which suggests that the interveinal yellowing and leafroll symptoms present in Saudi samples are probably a result of PeLRCV infection, as BPEV is not involved in symptoms development in host plants (Okada et al. 2011).

PeLRCV was transmitted successfully by *A. gossypii* in greenhouse conditions but the infection was limited to a relatively small number of plant species. However, we cannot exclude the possibility of any of these nonhosts being susceptible under different experimental conditions or with the use of another vector species. The laboratory host range found for PeLRCV is different from the host range reported for the other pepper-infecting poleroviruses. It has broader host range than PeVYV and narrower than PYLCV. PeLRCV can infect *C. annuum*, *C. amaranticolor*, *C. melo*, *D. stramonium*, and *S. nigrum*. PeVYV's host range consists only of *C. annuum* (and other *Capsicum* species) and *S. nigrum* (Knierim et al. 2013; Yonaha et al. 1995), while PYLCV can infect *C. annuum*, *C. amaranticolor*, *C. pepo*, *D. stramonium*, *G. globosa*, *N. benthamiana*, *N. clevelandii*, *P. hybrida*, and *P. floridana* (Dombrovsky et al. 2010). Moreover, there are differences in symptomatology even in common hosts, such as *C. amaranticolor* where the infection by PeLRCV remains asymptomatic, whereas PYLCV causes yellowing and small local lesions.

Similar to the other pepper-infecting poleroviruses, PeLRCV induces interveinal yellowing and upward rolling of leaf blades, shortening of stem internodes, stunting, and malformation of fruit. Field observations revealed that PeLRCV-infected plants grown in shaded patches of the greenhouses did not show yellowing and leafroll symptoms. Similar cases were reported for different poleroviruses and luteoviruses, suggesting that high light intensity plays an important role in symptom expression (Rochow and Duffus 1981).

The three-year survey (2014 to 2016) revealed the endemic nature of PYD, which is predicted to become a major constraint of bell pepper production in Saudi Arabia in the coming years. An extensive epidemiological study is necessary to identify the natural host range of the virus and the role of other aphid species frequently found in Saudi Arabia, such as *M. persicae* and *A. fabae*, in vectoring the virus.

Fig. 8. Agarose gel (1%) showing RT-PCR results of host range experiment of Pepper leafroll chlorosis virus. Positive results were observed for *Capsicum annuum*, *Cucumis melo*, *Solanum nigrum*, *Chenopodium amaranticolor*, and *Datura stramonium* (lanes 1, 4, 6, 10, 16), respectively. No RT-PCR product was amplified from other host plants including: *Amaranthus viridis*, *Cucurbita pepo*, *Citrullus lanatus*, *Lagenaria siceraria*, *Solanum lycopersicum*, *S. melongena*, *Nicotiana tabacum*, *Petunia hybrida*, *Vicia faba*, or *Vigna radiata* in addition to uninfected bell pepper as a negative control (lane 8). The arrow indicates the expected size (693 bp). Lane M represents Gene Dircx 100 bp DNA molecular weight marker (pictured on the left).

These will provide information about the natural reservoirs and elucidate the role of hosts like *D. stramonium* and *S. nigrum*, which are common arable weeds in Saudi Arabia. Finally, other cultivated Solanaceae and Cucurbitaceae must be assessed since PeLRCV has a broader host range than the other pepper poleroviruses, it can infect *C. melo*, and it could be related to disease development in other cultivated hosts.

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