AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

CELLULAR RESPONSE TO INFECTION

Transmission of a New Polerovirus Infecting Pepper by the Whitefly *Bemisia tabaci*

Saptarshi Ghosh,[a] Surapathrudu Kanakala,[a] Galina Lebedev,[a] Svetlana Kontsedalov,[a] David Silverman,[b] Tamar Alon,[b] Neta Mor,[b] Noa Sela,[c] Neta Luria,[c] Aviv Dombrovsky,[c] Munir Mawassi,[c] Sabrina Haviv,[c] Henryk Czosnek,[d] Murad Ghanim[a]

[a]Department of Entomology, Volcani Center, ARO, Rishon LeZion, Israel

[b]Agricultural Extension Service, Ministry of Agriculture & Rural Development, Rishon LeZion, Israel

[c]Department of Plant Pathology and Weed Research, Volcani Center, ARO, Rishon LeZion, Israel

[d]Institute of Plant Sciences and Genetics in Agriculture, Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot, Israel

ABSTRACT Many animal and plant viruses depend on arthropods for their transmission. Virus-vector interactions are highly specific, and only one vector or one of a group of vectors from the same family is able to transmit a given virus. Poleroviruses (*Luteoviridae*) are phloem-restricted RNA plant viruses that are exclusively transmitted by aphids. Multiple aphid-transmitted polerovirus species commonly infect pepper, causing vein yellowing, leaf rolling, and fruit discoloration. Despite low aphid populations, a recent outbreak with such severe symptoms in many bell pepper farms in Israel led to reinvestigation of the disease and its insect vector. Here we report that this outbreak was caused by a new whitefly (*Bemisia tabaci*)-transmitted polerovirus, which we named Pepper whitefly-borne vein yellows virus (PeWBVYV). PeWBVYV is highly (>95%) homologous to *Pepper vein yellows virus* (PeVYV) from Israel and Greece on its 5′ end half, while it is homologous to *African eggplant yellows virus* (AeYV) on its 3′ half. Koch's postulates were proven by constructing a PeWBVYV infectious clone causing the pepper disease, which was in turn transmitted to test pepper plants by *B. tabaci* but not by aphids. PeWBVYV represents the first report of a whitefly-transmitted polerovirus.

IMPORTANCE The high specificity of virus-vector interactions limits the possibility of a given virus changing vectors. Our report describes a new virus from a family of viruses strictly transmitted by aphids which is now transmitted by whiteflies (*Bemisia tabaci*) and not by aphids. This report presents the first description of polerovirus transmission by whiteflies. Whiteflies are highly resistant to insecticides and disperse over long distances, carrying virus inoculum. Thus, the report of such unusual polerovirus transmission by a supervector has extensive implications for the epidemiology of the virus disease, with ramifications concerning the international trade of agricultural commodities.

KEYWORDS *Bemisia tabaci*, circulative, polerovirus, transmission, aphid

Plant viruses have either an RNA or a DNA genome, with some of the viruses causing serious economic losses (1). About 75% of plant viruses are transmitted by insect vectors (2). Transmission can be either noncirculative (the virus remains stylet/foregut-borne) or circulative (the virus translocates in the digestive tract and moves to the hemolymph and then to the salivary glands) (3). Virus-vector interactions are highly specific; only one insect species or a one member of a group of species from the same insect family is responsible for transmission of all plant viruses within a genus level (3). Although intergeneric viruses within a virus family are known to utilize diverse insect vectors for transmission, many virus families are also restricted to a specific insect

Citation Ghosh S, Kanakala S, Lebedev G, Kontsedalov S, Silverman D, Alon T, Mor N, Sela N, Luria N, Dombrovsky A, Mawassi M, Haviv S, Czosnek H, Ghanim M. 2019. Transmission of a new polerovirus infecting pepper by the whitefly *Bemisia tabaci*. J Virol 93:e00488-19. https://doi.org/10.1128/JVI.00488-19.

Editor Anne E. Simon, University of Maryland, College Park

Address correspondence to Murad Ghanim, ghanim@agri.gov.il.

Received 22 March 2019
Accepted 26 April 2019
Accepted manuscript posted online 15 May 2019
Published 17 July 2019

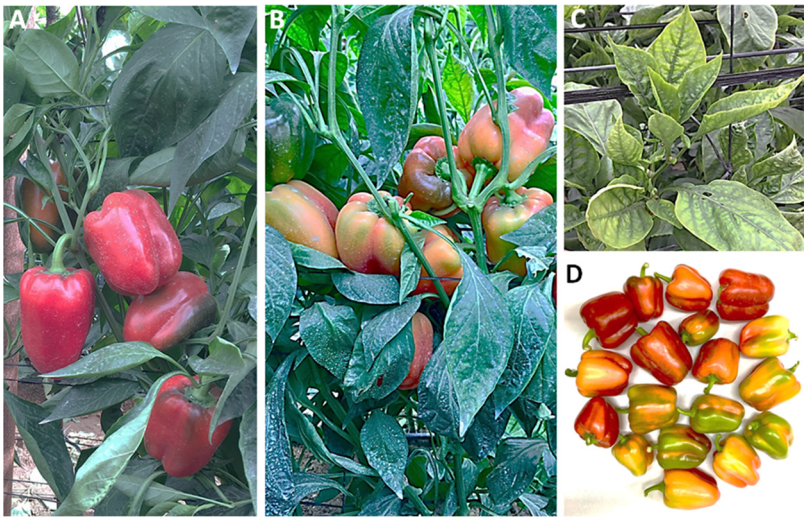

FIG 1 Symptoms caused by the newly identified PeWBVYV in pepper. The major damage is the discoloration of the fruits, as shown in panels B and D, compared to normal fruits (A), as well as the reduction in fruit size and change in fruit shape (D). (C) Infected plants with leaves showing yellowing and upward curling and stunted growth compared to the normal green leaves.

vector. *Luteoviridae*, a family of phloem-restricted viruses, comprised of three genera (*Luteovirus*, *Polerovirus*, and *Enamovirus*), is one such example wherein all viruses within the family are exclusively transmitted by aphid species (4). Virus-vector specificity is a major factor limiting the emergence of new virus-vector associations and thus had been used as a major parameter for classification of plant viruses in the past.

The genus *Polerovirus* includes at least 31 virus species (International Committee on Taxonomy of Viruses [ICTV] 2018b release) which are transmitted to diverse plants by aphid species in a circulative and persistent manner (5). Polerovirus particles are nonenveloped and icosahedral in shape and are 23 to 30 nm in diameter. The genome consists of monopartite, linear, single-stranded positive-sense RNAs, ranging in size from 5.3 to 6.2 kb. The genome is organized into seven open reading frames (ORFs) (ORF0 to ORF5 and ORF3a) encoding proteins P0 to P5 and P3a, respectively. Poleroviruses are prone to recombination among themselves or with viruses belonging to different families (6, 7). The frequency of recombination events has made poleroviruses the fastest growing genus among the *Luteoviridae*, resulting in an increase in the number of reported species from 9 (8) to 31 (ICTV 2018b release) in 15 years.

When a polerovirus infects pepper plants, it induces disease symptoms that include interveinal yellowing of leaves, shortened internode length, upward leaf curling, and discoloration of mature fruits. These symptoms, initially described in Japan (9) and Israel (10), were associated with a polerovirus recently named *Pepper vein yellows virus* (PeVYV) (11). At least seven different polerovirus-like species associated with similar symptoms in pepper have been described worldwide. They have been named PeVYV 1 to 5 (12) and two recently described viruses from Greece (13) and Saudi Arabia (14) are referred to in this study as PeVYV-6 and PeVYV-7, respectively. Typical of poleroviruses, the PeVYVs are strictly transmitted by aphid species (*Aphis gossypii* and *Myzus persicae*) in a persistent circulative manner (10).

Since 2016, there has been a disease outbreak affecting bell peppers cultivated in the Jordan Valley, Israel. During two consecutive seasons, all the plants in several net houses were affected, causing heavy economic losses. The disease symptoms resembled those caused by an aphid-transmitted polerovirus previously reported in Israel, currently named PeYVY-2 (Fig. 1). However, extension workers and farmers reported low numbers of aphids in the net houses. Monitoring insects with yellow sticky traps at intervals of 15 days, from mid-September 2017 to mid-January 2018, confirmed the low abundance of aphids in the net houses containing severely infected bell peppers.

Hence, the disease outbreak could not be associated with aphids, the only known vector of poleroviruses. On the other hand, high numbers of whiteflies (*Bemisia tabaci*) were conspicuous in these net houses. This led us to reinvestigate the disease and search for alternative vectors.

In this study, we present evidence associating this disease outbreak in pepper with a new polerovirus species and report its complete genome sequence. Most interestingly, for the first time we demonstrate transmission of a polerovirus by the whitefly *B. tabaci*. Koch's postulates and transmission with *B. tabaci* were also confirmed with an infectious clone. We therefore name this virus Pepper whitefly-borne vein yellows virus (PeWBVYV).

RESULTS

The pepper disease with polerovirus-like symptoms is transmissible not by aphids but by whiteflies. To find out whether whiteflies and aphids were able to transmit the putative polerovirus, symptomatic plants were removed from the greenhouse, potted, and used as the virus source for transmission experiments with aphids and whiteflies. After an acquisition access period of 7 days, whiteflies and aphids were caged for 48 h with noninfected pepper plants (10 insects each in a clip cage per plant). Forty percent (18 of 45) of the plants inoculated with whiteflies presented typical disease symptoms. Reverse transcription-PCR (RT-PCR) analyses performed with polerovirus-specific primers showed that all the symptomatic plants contained polerovirus RNA (Fig. 2A to C). Inoculation by continuous exposure with higher whitefly numbers mimicking the net house conditions led to about 71% (17 of 24) of the plants developing symptoms, all of which contained polerovirus RNA (Fig. 2D). When the aphids (*M. persicae*) were given an acquisition period of 48 h of access to the same virus source, followed by continuous inoculation access to virus-free pepper seedlings for up to 45 days, none of the plants became infected or developed disease symptoms (0 of 30) and all 30 of the plants tested negative for polerovirus. Although only the whiteflies were able to transmit the polerovirus, both the aphids and the whiteflies tested positive for the virus by RT-PCR following access to infected pepper plants from the greenhouse. In contrast, PeVYV-2 could not be transmitted by *B. tabaci* under conditions of lifelong inoculation access to five virus-free pepper plants after acquisition access of 48 h (Fig. 2E).

A new polerovirus is associated with the disease outbreak in pepper. The polerovirus-like identity of the virus associated with the disease outbreak from eight pepper net houses in Jordan Valley in Israel was confirmed by sequencing the RT-PCR fragment obtained using universal polerovirus primers. Results indicated the presence of a virus distinct from PeVYV-2, the previously described pepper-infecting polerovirus from Israel. In view of the results demonstrating transmission of a polerovirus by whiteflies and the loss of transmissibility by aphids, we subjected the whitefly-transmitted polerovirus to Illumina sequencing. The *de novo*-assembled sequence was validated by RT-PCR amplification, cloning, and sequencing of three overlapping genome fragments of 4,179, 778, and 1,208 bp with virus-specific primers (Fig. 3). Sequences at the termini were obtained by rapid amplification of cDNA ends (RACE) PCR. The final assembly revealed a 5,925-nucleotide-long genome (see below for GenBank accession number) with an organization typical of poleroviruses. It had seven ORFs (Fig. 3A), including ORF0 and ORF4 (which are characteristic of poleroviruses), and lacked the 3′ proximal ORF6 found in luteoviruses. The 5′ and 3′ ends of the genome typically started with ACAAA and ended with GT. We named this virus Pepper whitefly-borne vein yellows virus (PeWBVYV).

Comparison of the amino acid sequences of the respective proteins with the other seven pepper-infecting poleroviruses and related viruses indicated a recombination event (Fig. 4). Proteins encoded by the 5′ half of the genome (P0, P1, and the P1-P2 fusion protein) were mostly identical with those of PeVYY-2 and PeVYV-6, whereas proteins translated from the 3′ half (P3 to P5) were more nearly identical to those of *African eggplant yellowing virus* (AeYV), which was recently reported from Benin and

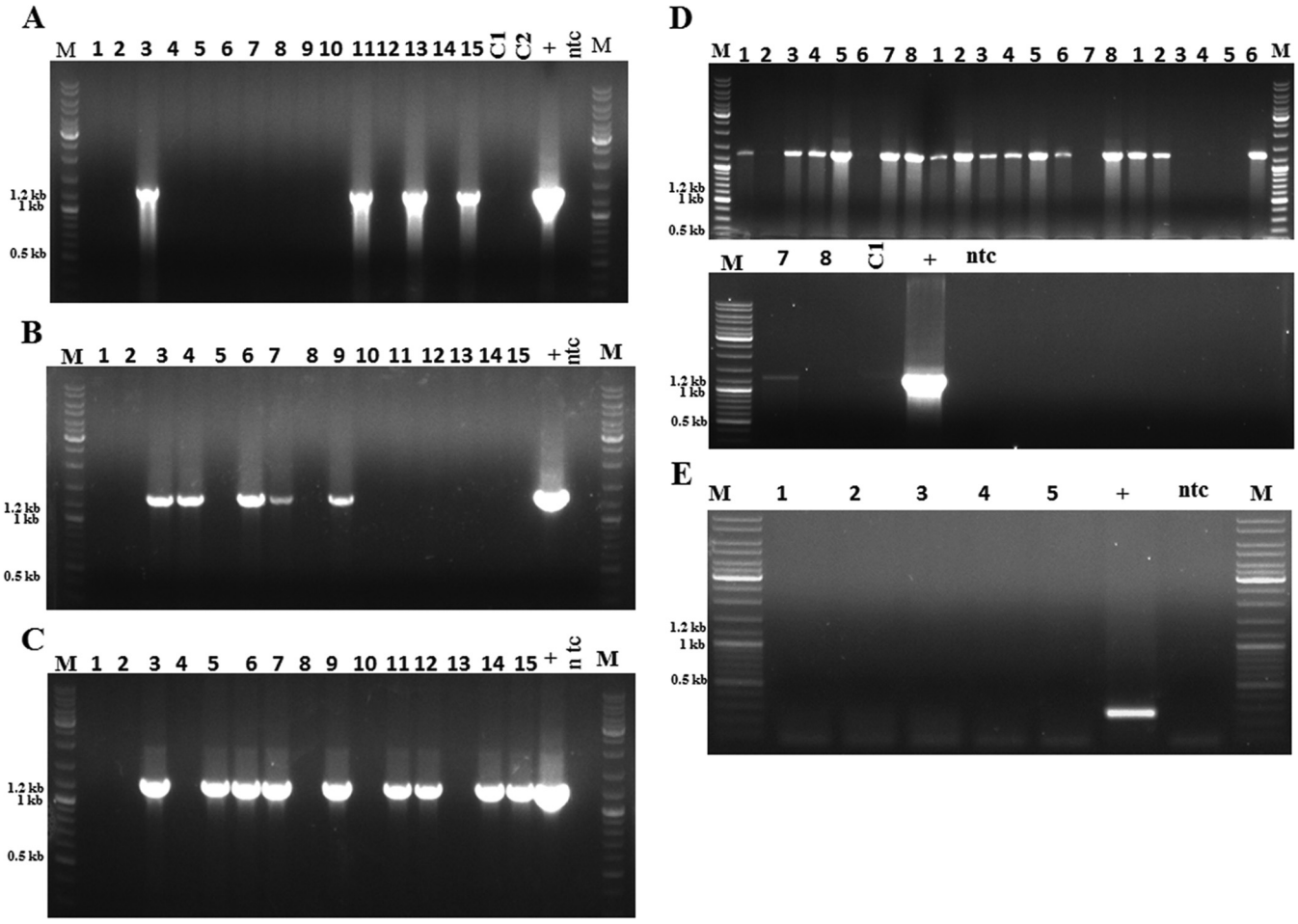

FIG 2 (A to C) Raw results of transmission of PeWBVYV as determined by RT-PCR from plants given an inoculation access period of 48 h with 10 viruliferous whiteflies per plant using clip cages in three replicates, with 15 plants each. Panels A, B, and C represent the first, second, and third replicates, respectively. "C1" and "C2" represent control plants inoculated with nonviruliferous whiteflies, while "+" and "ntc" denote positive controls and no-template controls, respectively. (D) Raw results of transmission of PeWBVYV as determined by RT-PCR from pepper plants given continuous inoculation access with ~30 viruliferous whiteflies per plant in three replicates (8 plants each). "C1" refers to a control plant inoculated with nonviruliferous whiteflies, and "+" denotes a positive control. (E) Control experiment for testing the inability of *B. tabaci* to transmit PeVYV-2 after acquiring the virus for 48 h from infected plants and lifelong transmission to five healthy plants (lanes 1 to 5) compared to a positive control (+). M, molecular weight markers.

Mali in Africa (15). Recombinant analysis of PeWBVYV using the RDP4 platform also indicated a single recombination event at nucleotide position 2826, with PeVYV-2 and AeYV as the minor parent and major parent, respectively (Fig. 5), using the GENECONV (*P* value of 8.48 E−175), RDP (9.77 E−156), BootScan (1.89 E−164), MaxChi (5.89 E−59), Chimera (4.06 E−59), SiScan (5.85 E−77), and 3seq (1.86 E−14) methods. The longest protein translated from the 3′ end of the genome, representing the readthrough domain (RTD) of PeWBVYV, was different from most of the PeVYVs (39.6% to 46.3% similarity), except for PeVYV-7 (92.3%; partial sequence) and AeYV (89.2%). Interestingly, the N-terminal half (254 amino acids from the end of the coat protein) of the RTD of the currently described PeWBVYV was nearly identical (92% to 95.4%) to both PeVYV-7 and AeYV, but the sequence was less similar (80% to 83%) at the C-terminal half (254 amino acids). A recently described polerovirus (PeVYV-7) infecting pepper from Saudi Arabia had high (>90%) amino acid sequence identity with almost all the proteins (except P3a) encoded by PeWBVYV, although only a partial sequence of the readthrough domain (RTD) is available for the former. The smallest encoded protein, P3a, was the most variable, with 78% to 86.5% identity with other closely related viruses (Fig. 4). In addition, P3a is initiated from a non-AUG start codon, namely, CUG for PeWBVYV and AeYV, and is initiated at AUA for the other PeVYV species. Among the three noncoding

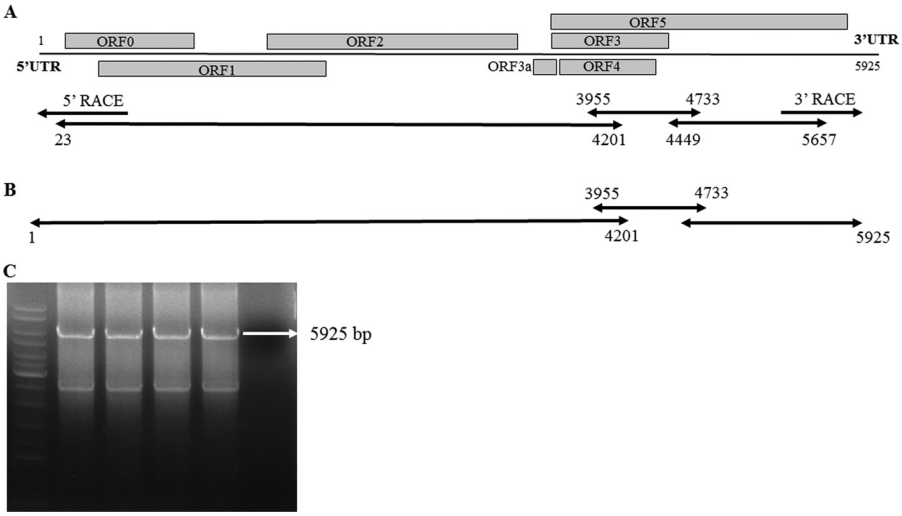

FIG 3 Genetic organization of PeWBVYV. (A) PeWBVYV ORFs and validation strategy using RT-PCR amplification. UTR, untranslated region. (B) Strategy for assembly of cDNA corresponding to the PeWBVYV RNA genome from overlapping fragments by overlap extension PCR. (C) PCR amplification of cDNA corresponding to the PeWBVYV RNA genome by overlap PCR.

regions (NCR), the 5′ NCR (50 bp) was 100% identical to PeVYV-2, the intergenic region (78 bp) was less (80.2% to 83.7%) identical to all the PeVYVs and most (90.14%) similar to AeYV, and the 3′ NCR (402 bp) was highly (80%) divergent from all the others and shared homology only with AeYV. No additional AUG-initiated ORFs encoding proteins homologous to P6 and P7 of the *Potato leaf roll virus* could be found in the 3′ NCR or within ORF5 of PeWBVYV.

Constructing an infectious clone of the new polerovirus to fulfill Koch's postulate. An infectious clone was constructed to confirm that PeWBVYV causes the symptoms on pepper and that the virus is transmitted by whiteflies but not by aphids. Attempts to obtain a full-length clone by direct PCR amplification failed. Therefore, a full-length (5,925-bp) PeWBVYV cDNA clone was accurately assembled from three

Virus species	Accession Number	5' NCR	P0	P1	P1-P2	Intergenic NCR	P3a	P3	P4	P5 fusion	N-P5 254 aa	C-P5 254 aa	3' NCR
PeWBVYV													
PeVYV-1	AB594828	93.48	84.55	91.2	89.6	83.1	83.78	79.58	61.64	44.98			
PeVYV-2	HM439608	100	98.78	97.92	95.88	81.69	78.38	80.63	60.96	39.61			
PeVYV-3	KP326573	93.48	89.84	95.36	92.88	83.1	83.78	79.58	62.33	46.24			
PeVYV-4	KU999109		84.55	90.08	90.35	83.1	83.78	79.58	60.27	44.98			
PeVYV-5	KY523072	97.83	86.18	92.96	92.5	81.69	83.78	80.1	59.59	45.88			
PeVYV-6	LT559484	100	97.97	98.72	96.81	81.69	83.78	80.1	59.59	44.98			
PeVYV-7	LT220496	97.83	93.9	96.32	94.66	80.28	83.78	95.29	95.21	92.29*	95.44	82.93*	
AeYV	NC_010732	63.04	40.65	62.08	71.42	90.14	86.49	92.15	86.99	89.25	92.12	80.49	80
TVDV	NC_034207	82.61	76.83	79.04	84.25	81.69	83.78	79.58	60.27	39.43			

FIG 4 Amino acid (aa) sequence identities of the translated proteins and nucleotide identities of the noncoding regions (NCR) of PeWBVYV with those of other closely related virus species. Blank cells and asterisks (*) indicate divergent sequences and partial sequence availability, respectively. Increasing levels of saturation of red and green denote high and low amino acid similarity, respectively.

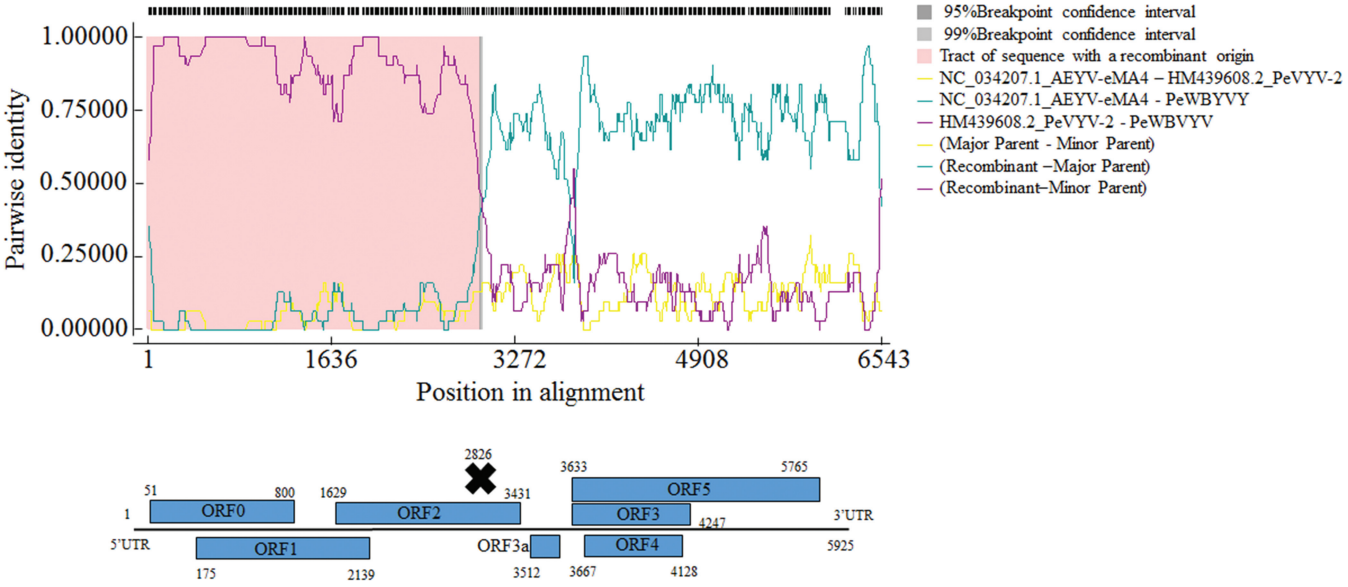

FIG 5 Recombination (RDP) and genetic map of PeWBVYV showing major (AeYV) and minor (PeVYV-2) parents (top) and the transition in the genome sequence at the breakpoint (X; 2,826 bp) at ORF2 (bottom).

overlapping fragments obtained by RT-PCR (from nucleotides 1 to 4201, 3955 to 4733, and 4449 to 5925) (Fig. 3B and C), cloned in binary vector pJL89, and transformed into *Agrobacterium tumefaciens*. The PeWBVYV-containing agrobacterium was delivered to *Nicotiana benthamiana* plantlets by infiltration and to pepper seedlings by injection. All the agroinfected plants showed mild yellowing of leaves 20 days after the treatment. Infection by PeWBVYV of both *N. benthamiana* and pepper was confirmed by Northern blotting (Fig. 6A) and by RT-PCR of total leaf RNA. The level of virus accumulation at

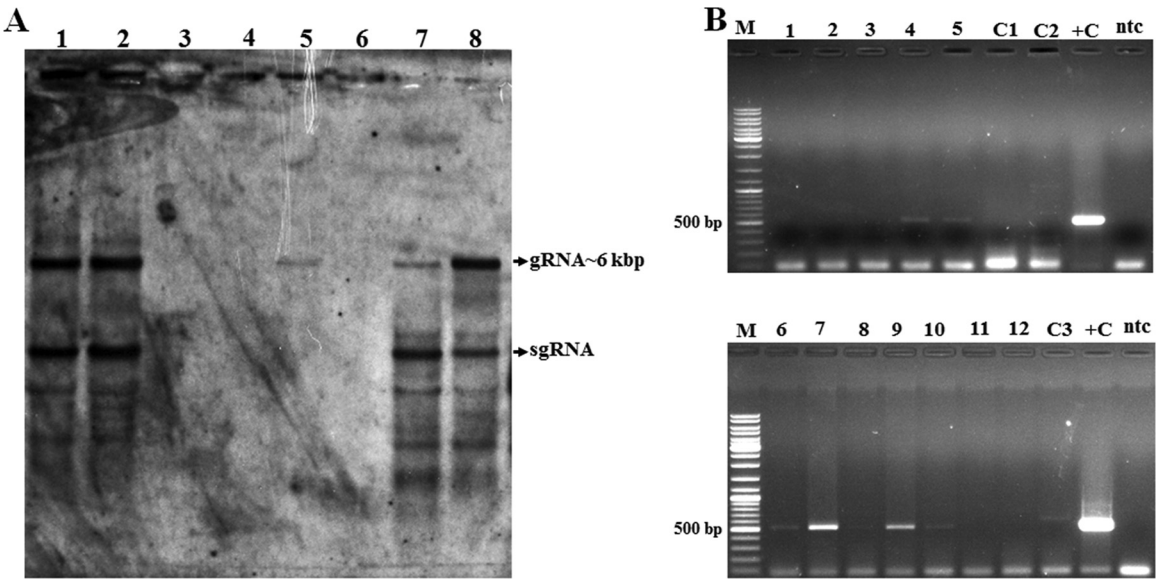

FIG 6 Northern blot analysis of *N. benthamiana* and pepper plants inoculated with the PeWBVYV infectious clone. (A) Lanes 1 and 2, *N. benthamiana* inoculated with the PeWBVYV clone; lanes 3 and 4, *N. benthamiana* inoculated with the binary vector pJL89 alone; lane 5, pepper inoculated with PeWBVYV clone; lane 6, pepper inoculated with the binary vector pJL89 alone; lanes 7 and 8, pepper naturally infected with whiteflies (positive control); gRNA, genomic RNA; sgRNA, subgenomic RNA. (B) RT-PCR confirmation of transmission of PeWBVYV infectious clone by *B. tabaci*. Lanes 1 to 12 denote pepper plants inoculated with *B. tabaci* after a 48-h acquisition access period on pepper plant agroinoculated with PeWBVYV. C1 and C2, pepper plants caged with *B. tabaci* after 48 h of rearing on virus-free pepper plants inoculated with pJL89 alone; +C, pepper plant infected with the PeWBVYV infectious clone; ntc, no-template control; M, molecular weight markers.

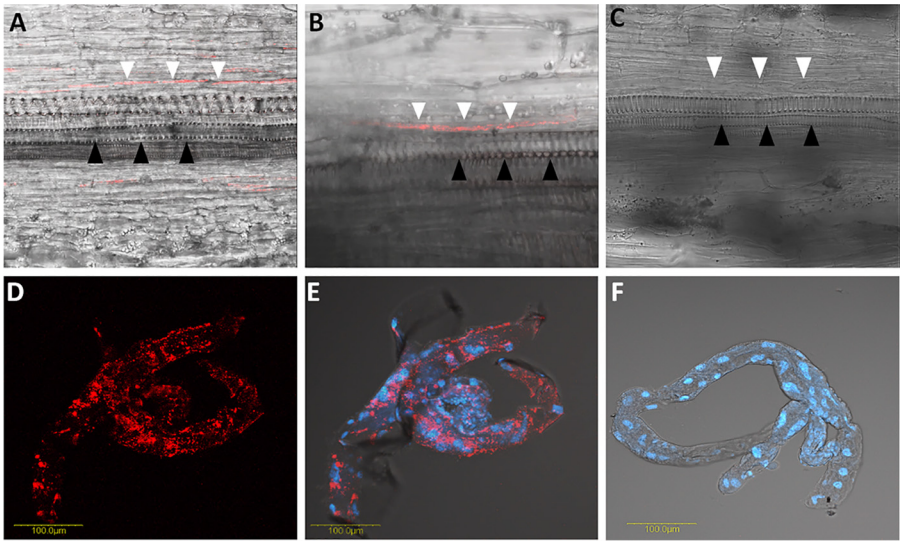

FIG 7 (A to C) Localization of PeWBVYV (red) in the phloem of naturally infected pepper plants (A) and in *N. benthamiana* plants agroinoculated with the PeWBVYV infectious clone (B) compared to control uninfected plant hybridized with the same probe (C). White arrowheads indicate virus (red) inside phloem sieve elements and black arrowheads indicate xylem. (D to F) Localization of PeWBVYV (red) inside midguts of *B. tabaci* after acquisition from naturally infected (D) and agroinfected (E) pepper plants compared to acquisition from a control uninfected plant hybridized with the same probe (F). Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole; blue).

20 days postinfection (dpi) was lower in the pepper plants, possibly due to host RNA silencing mechanisms, as no construct designed to suppress plant silencing mechanisms was used. The exclusive presence of viral RNA in phloem cells of naturally infected pepper plants (Fig. 7A) and in *N. benthamiana* agroinfected with the infectious clone (Fig. 7B and C) was confirmed by fluorescence *in situ* hybridization (FISH). Whitefly acquisition of PeWBVYV from plants infected with the infectious clone and its transmission to test plants were demonstrated. Approximately 120 whiteflies were caged for 48 h with agroinfected pepper plants. Acquisition of PeWBVYV by *B. tabaci* in dissected midguts from adult whitefly females was confirmed by FISH (Fig. 7D to F). The insects were transferred *ad libitum* to five virus-free pepper plants. Inoculated plants were tested for the presence of virus after 20 days; 6 (50%) of the 12 plants tested contained detectable viral RNA (Fig. 6B). Koch's postulates were therefore fulfilled for the new PeWBVYV by the use of the infectious clone to confirm transmission by whiteflies.

Virus detection and distribution in Israel. The origin of PeWBVYV is not known. In view of that, a multiplex PCR assay was designed for simultaneous detection of PeWBVYV and the previously described PeVYV-2 from Israel (Fig. 8). This assay was used to survey the distributions of the two viruses in major pepper-growing areas of Israel during two successive cropping seasons (January to April 2017 and 2018). Leaf/fruit samples from symptomatic and nonsymptomatic pepper plants were collected from net houses and greenhouses in the Jordan Valley area of Israel, the Arava Valley, and the northern coastal area. PeWBVYV was detected in the Jordan Valley and coastal area but was absent in the Arava Valley (Fig. 9). Infection with the two viruses in the same plant was not found.

DISCUSSION

A new disease outbreak with severe symptoms has caused heavy losses to bell pepper cultivation in Israel due to the complete nonmarketability of affected fruits with discoloration and insipid taste. These symptoms, although similar to those caused by the previously described pepper-infecting polerovirus species PeVYV-2, could not be correlated with the abundance of its known aphid vector (*M. persicae*). In this report, we associate the cause of this outbreak with a new polerovirus species, PeWBVYV, which

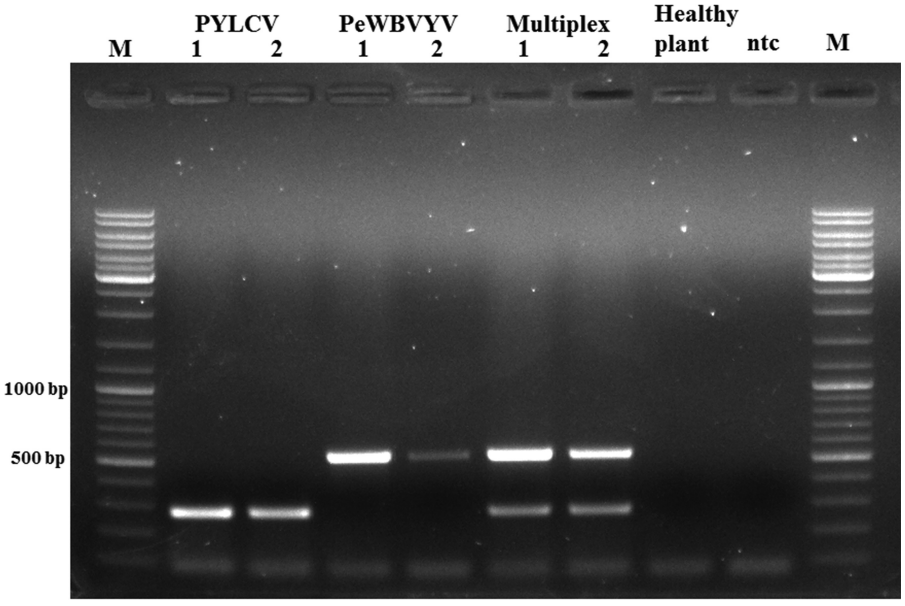

FIG 8 Multiplex detection of PeWBVYV and PeVYV-2 showing single detection of each virus alone and multiplex detection of both viruses in the same reaction. ntc, no-template control; M, molecular weight markers.

is not transmitted by aphids, the known vectors for poleroviruses. PeWBVYV, however, has a genome organization similar to that typical of poleroviruses, with six ORFs, starting and ending with characteristic ACAAA and GT sequences, respectively, and was shown to be phloem bound in infected pepper leaves. Surprisingly, this virus was found to be transmitted by the whitefly *B. tabaci*, a well-known supervector of viruses. Whiteflies are known to transmit DNA viruses (genus *Begomovirus*) as well as RNA viruses belonging to the genera *Crinivirus*, *Ipomovirus*, and *Torradovirus* but not to the genus *Polerovirus* (16). Inoculation of PeWBVYV with 10 viruliferous whiteflies per plant in clip cages for 48 h resulted in a 40% infection rate, while continuous exposure with higher whitefly numbers led to a 71% transmission success rate. In control experiments, the previously reported PeVYV-2 from Israel could not be transmitted by whiteflies and PeWBVYV could not be transmitted by aphids, suggesting that the latter is the first described polerovirus transmitted by whiteflies.

Existence of transmission by diverse insect vectors is a common phenomenon within many plant virus families such as *Geminiviridae*, *Caulimoviridae*, *Potyviridae*, and *Rhabdoviridae*, although they are demarcated into vector-specific genus categories. The list of such varied vectors continues to expand with improvements in techniques for virus characterization and classification. A recent example is *Alfalfa leaf curl virus*, the first reported aphid-transmitted virus within the *Geminiviridae* (17). The altered transmission of PeWBVYV by whiteflies, too, initially suggested that PeWBVYV represented and added example of such phenomenon within *Luteoviridae*. Species demarcation within poleroviruses is based on the criterion of a difference in the level of amino acid sequence identity of any gene product higher than 10%. PeWBVYV narrowly meets the species criteria, with only 10.75% and 13.51% divergence from AeYV on the RTD and P3a proteins, respectively. Even PeVYV-7 was more than 90% identical to PeWBVYV in amino acid composition for most of the proteins (except P3a), although only a partial sequence of PeVYV-7 RTD is available. Thus, the high similarity of the PeWBVYV genome to the genomes of other known aphid-transmitted poleroviruses indicates that PeWBVYV does not belong to a new group within the *Luteoviridae*. Therefore, PeWBVYV could be the first described whitefly-transmitted virus within the polerovirus genus.

In virus-vector interactions, the capsid protein (CP) plays a major role in vector specificity. The luteovirid capsids are composed of the following two proteins, both

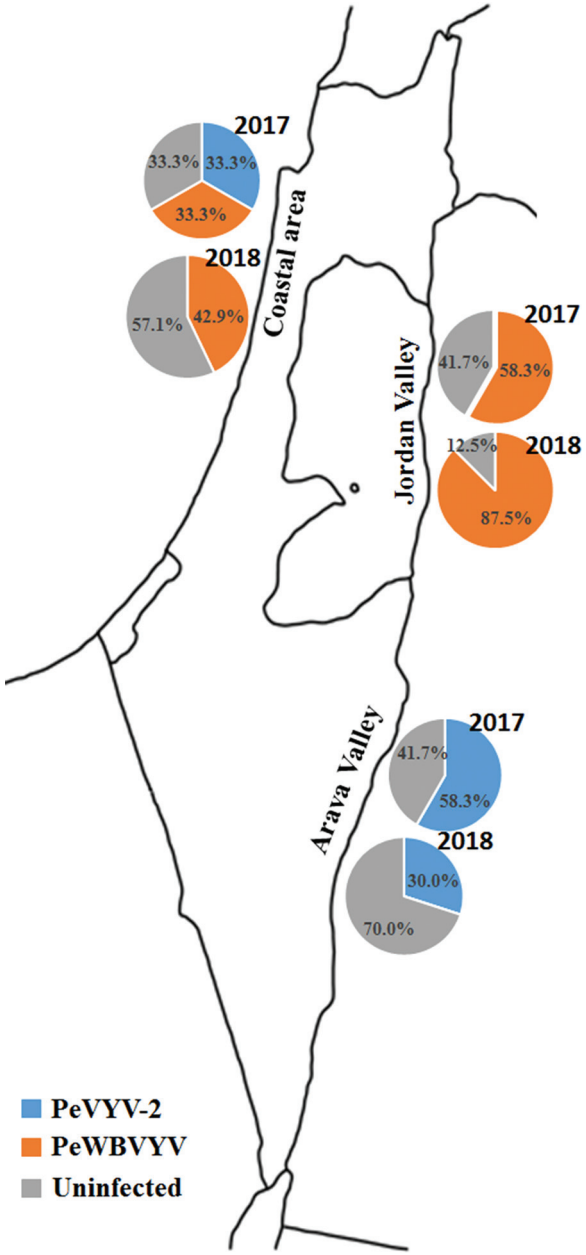

FIG 9 Distribution of PeVYV-2 and PeWBVYV in Israel, sampled from three major pepper-growing areas (Jordan Valley, Arava Valley, and the coastal area) from January to April 2017 and 2018. Pie charts indicate the incidence of PeVYV-2 and PeWBVYV detection in two successive seasons (2017 and 2018). The total numbers of plants tested in the two seasons in each of the specified locations were as follows: for Jordan Valley, 24 and 36, respectively; for the Arava Valley area, 10 and 36, respectively; for the coastal area, 7 and 9, respectively.

involved in transmission by aphids: the major CP (of about 22 kDa) and the minor CP (readthrough domain or RTD of about 55 kDa). RTD is responsible for vector specificity (18). Point mutations in the RTD impair transport of virions in the gut cells (15) and uptake by the accessory salivary glands (19–22). Moreover, RTD interacts with GroEL produced by aphid endosymbionts, likely protecting the virus from destruction in the hemolymph (23). The specificity of poleroviruses with respect to their aphid vectors is determined by the N-terminal region of the RTD (18, 23) and by its specificity in binding to the GroEL chaperonin of the aphid bacterial symbiont. Despite the preexisting knowledge on polerovirus-aphid interactions, we cannot explain the nontransmissibil-

ity of PeWBVYV by aphids. PeWBVYV, although similar to PeVYV-7 in most protein sequence, is unlike the latter in aphid transmission. PeVYV-7 infections in pepper, like the infections by all other PeVYVs, were perfectly transmissible by aphids (14). However, the known vector determining the RTD region of PeWBVYV had low homology with all other closely related viruses except PeVYV-7 and AeYV. Much to our surprise, both PeVYV-7 and AeYV were more nearly identical at the N-terminal half of the RTD (92% to 95.4% identity) of PeWBVYV than at the C-terminal half (80% to 83%). Thus, the high similarity of the amino acid sequences at the N-terminal RTD of PeWBVYV and PeVYV-7 makes the aphid nontransmissibility of the former perplexing. The vector of AeYV is as yet unknown; however, the incidence of AeYV likely correlates with aphid abundance (24), and the vector is thus assumed to be aphid such as is the case with all other poleroviruses. More importantly, the genome portion governing whitefly transmission of PeWBVYV remains unknown. Future availability of sequences at the terminal end of the PeVYV-7 genome and its comparison with the genomes of PeWBVYV and AeYV would shed more light on the role of RTD with regard to the insect vectors.

Genomic RNA recombination leading to the formation of chimeric molecules from noncontiguous parental genomes is pivotal for the emergence of new viruses. Exchanges of long nucleotide sequences are particularly common in *Luteoviridae* and can result in drastic changes in viral phenotypes (6). Poleroviruses undergo sympatric speciation, with frequent recombination events between viruses infecting the same host species in a small geographical area (7, 25). The PeWBVYV genome, too, appears to represent the result of an event of recombination. The 5′ half of the PeWBVYV genome shares high identity with those of a previously described pepper-infecting polerovirus (PeVYV-2) from Israel (26) and a recently described species (PeVYV-6) from Greece (13). The 5′ nontranslated region (5′NTR) of the PeWBVYV genome was 100% similar to that of PeVYV-2/6 and the encoded proteins P0, P1, and P1-P2 were also identical in the ranges of 98% to 98.8%, 97.9% to 98.7%, and 95.9% to 96.8%, respectively. The 3′ half, however, was much more distant from that of PeVYV-2/6 but was more homologous with that of a polerovirus infecting pepper (PeVYV-7) from Saudi Arabia (14) and with that of AeVY, a polerovirus infecting eggplants in Africa (24). AeYV, though, has been reported from Africa to infect both eggplants (Mali) and, to a much lesser extent, pepper (Benin) (24). Recombinant analysis of PeWBVYV using the RDP platform indicated a clear breakpoint toward the C-terminal end of the RdRp fusion protein (P1-P2), with AeYV and PeVYV-2 as the major and minor parents, respectively. Whether the exclusive whitefly transmissibility of PeWBVYV was a result of recombination between two aphid-transmitted viruses is unknown. Moreover, the place of origin of PeWBVYV and its route of entry into Israel are unknown. The relative similarity of PeWBVYV to PeVYV-7 and its high incidence in the Jordan Valley area indicate a mid-Eastern route of entry, possibly via Jordan. A further indication that the virus came from an external source was the fact that PeWBVYV was not detected in the Arava Valley, the major pepper-growing region in Israel, in both surveyed seasons. Interestingly, coinfections by PeWBVYV and PeVYV-2 in the same pepper plant were never detected; such an absence of mixed polerovirus infections was observed previously (24). The dynamics of PeWBVYV and PeVYV-2 coinfection and interactions with the two insect vectors is compelling and will be investigated in future studies.

Transmission of a polerovirus by whiteflies constitutes an alarming threat to global trade of agricultural commodities. *B. tabaci*, a cryptic species complex considered a supervector of plant viruses, transmits over 300 viruses (16) and is thus one of the most important quarantine pests worldwide. Moreover, the MEAM1 species of whitefly used in this study is highly polyphagous, with a host range of over 500 plant species, and is resistant to multiple insecticides (16). It is also capable of dispersal of the viral inoculum to distances considerably greater than those seen with aphids. Thus, there is an urgent need for reassessments of the risks associated with poleroviruses. Further, the report of a new vector for a given virus would force growers to change pest management schemes and would thus have severe implications for global crop production.

TABLE 1 Primers and probes used in this study

Objective	Name	Position	Sequence	Product length (bp)
Sequence validation	VF3	23–43	5′-GCCCTTGCTAGTGATTTCTTC-3′	4,179
	AeYV-R	4,201–4,182	5′-CCGGCAGTTTTACCGCTAGT-3′	
	VF5	3,955–3,977	5′-TCTCAATGGTCAACATACGCT-3′	778
	VR5	4,733–4,713	5′-TGCTATAGTGACTTGGTCTGA-3′	
	VF4	4,449–4,469	5′-GCTGAAACCTTAGAGGTTCGC-3′	1,208
	VFR3	5,657–5,636	5′-TTTCTTAAGAGCTTCATACCGC-3′	
RACE PCR	dT adaptor		5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3′	
	Adaptor		5′-GACTCGAGTCGACATCG-3′	
	3′-RACE-F	5,510–5,519	5′-TAACTGGCGGTTCCGTAAAG-3′	
	5′-RACE-R	487–506	5′-GCGCAGGAATCTTGAAAACT-3′	
cDNA synthesis	3′-RT-R	5,925–5,906	5′-ACTGTATAAATCAGGAAAGA-3′	
	3′-PYLCV-RT-R	5,988–5,971	5′-CCCTAAGAATTATGATGC-3′	
PCR detection	AeYV-F	2,907–2,926	5′-ACATTGGACCTGAACCCCAC-3′	1,294
	AeYV-R	4,201–4,180	5′-CCGGCAGTTTTACCGCTAGT-3′	
Multiplex PCR detection	Common-F	3,194–3,214	5′-ACAACTGGAATTCTGCTCTCA-3′	
	PWBV-R	3,718–3,698	5′-ATTTGCTGAACTGGTCGAACT-3′	524
	PYLCV-R	3,464–3,441	5′-TGATGTATGCTTGGCTAGTTATA-3′	270
1st step overlap PCR	5′-F	1–24	5′-ACAAAATATACGAAGAGAGAGAGC-3′	4,201
	AeYV-R	4,201–4,182	5′-CCGGCAGTTTTACCGCTAGT-3′	
	VF5	3,955–3,977	5′-TCTCAATGGTCAACATACGCT-3′	778
	VR5	4,733–4,713	5′-TGCTATAGTGACTTGGTCTGA-3′	
	VF4	4,449–4,469	5′-GCTGAAACCTTAGAGGTTCGC-3′	1,476
	3′-R	5,925–5,902	5′-ACTGTATAAATCAGGAAAGACACT-3′	
2nd step overlap PCR	5′-Gibs-F	1–24	5′-GTTCATTTCATTTGGAGAGGACAAAATATACGAAGAGAGAGAGC-3′	
	3′-Gibs-R	5,925–5,902	5′-TGGAGATGCCATGCCGACCCACTGTATAAATCAGGAAAGACACT-3′	
Linearize vector	pJL89-F		5′-GGGTCGGCATGGCATCTC-3′	
	pJL89-R		5′-CCTCTCCAAATGAAATGAACTTCC-3′	
Colony PCR	pJL89-colF		5′-GACGTAAGGGATGACGCACA-3′	
	pJL89-colR		5′-CCTTAGCCATCCGAGTGGAC-3′	
Northern blot probe	3′-RACE-F	5,510–5,519	5′-TAACTGGCGGTTCCGTAAAG-3′	415
	3′-R	5,925–5,902	5′-ACTGTATAAATCAGGAAAGACACT-3′	
FISH	AeYV-R	4,201–4,182	Cy3 5′-CCGGCAGTTTTACCGCTAGT-3′	

MATERIALS AND METHODS

Virus samples, RT-PCR and sequencing, and RNA sequencing using an Illumina platform. Pepper (cv. Godzilla) leaf samples with and without symptoms were collected from 8 net houses in diverse locations in the Jordan Valley area of Israel. Total RNA was isolated using TRI reagent (Sigma-Aldrich) per the manufacturer's instructions. Treatment with DNase I and synthesis of first-strand cDNA with 2 μg RNA and random hexamers were done using a Maxima first-strand cDNA synthesis kit (Thermo Fisher Scientific). Diluted (1:10) cDNA was used as the template for PCR amplification with universal polerovirus primers Pol-G-F and Pol-G-R (27). PCR products were cloned into pGEM-T easy vector and sequenced from both directions (HyLabs Sequencing Service, Israel).

Total RNA was also sequenced on an Illumina HiSeq platform (Illumina Inc., USA). Quantification and purity analysis of the total RNA were performed by the use of a NanoDrop spectrophotometer. RNA fragmentation, synthesis of second-strand cDNA using random hexamers, adaptor ligation, PCR amplification of the cDNA library, and sequencing were done by the Beijing Genome Institute (BGI; Hong Kong). A total of 22,412,008 paired-end reads (100 bp each) were sequenced using an Illumina HiSeq platform. Trimming of adaptor sequences and low-quality sequences was done using Trimmomatic software (28). The assembly of the reads was done using an A5-assembly pipeline (29), and a total of 45,465 contigs were generated with N_{50} of 1222 bp. We used DIAMOND software (30) and the protein nonredundant NCBI database (31) to extract the sequences of the virus with high genome coverage (Fig. 2A).

The *de novo*-assembled sequence was validated by RT-PCR amplification and cloning and sequencing of genome fragments with virus-specific primers (Table 1). The genomic terminal sequences at both the 5′ and 3′ ends were obtained by RACE PCR as described previously (32). In brief, the 3′ end of the viral

RNA was polyadenylated using *Escherichia coli* poly(A) polymerase (NEB) and first-strand cDNA was synthesized using $(dT)_{17}$- adaptor primers (Table 1). The 3′ terminal end was PCR amplified from the cDNA pool using a virus-specific forward primer and the adaptor primer (Table 1). Similarly, the 5′ end of the cDNA, after purification by treatment with phenol/chloroform (6:3 [vol/vol]) was polyadenylated using terminal transferase (NEB) and PCR amplified using the $(dT)_{17}$- adaptor and an adaptor-specific and virus sequence-specific reverse primer (Table 1).

Genome sequence analysis. Sequence assembly from overlapping fragments and multiple-sequence alignments were done using MUSCLE algorithm and DNAMAN software. Nucleotide and protein sequence homologies were compared using a BLAST algorithm, and ORFs were determined using an ORF finder algorithm (NCBI). The P1-P2 (replicase) fusion protein was translated manually with a −1 frameshift at the slippery sequence (G GGA AAC) in the genome. Amino acid sequences translated from the ORFs of the seven known pepper-infecting poleroviruses and other closely related viruses (AeYV and TVDV) were aligned, and pairwise distances were calculated for comparisons. Events of recombination between the aligned viruses (except PeVYV-7 due to an incomplete genome sequence) were screened by the use of the RDP4 (Beta 4.97) package (33) using the RDP, GENECONV, Chimera, MaxChi, BootScan, SiScan, and 3Seq methods with default parameters.

Transmission with aphids and whiteflies. Aphids (*M. persicae*) were given acquisition access for 5 days on pepper plant leaflets manifesting symptoms collected from the net houses in the Jordan Valley and were released to 30 (3 groups of 10) disease-free pepper plants (cv. Godzilla) for lifelong exposure in insect cages and kept under controlled environmental conditions (±25°C, 60% relative humidity [RH], and a 14-h photoperiod). Aphids released to disease-free plants without acquisition access were used as negative controls. RNA was extracted from new leaves of the inoculated plants after 20 days, and aphids were tested for the virus by RT-PCR after the acquisition access period.

Similarly, a new whitefly (*B. tabaci*; MEAM1) (free of the endosymbiont *Rickettsia*) was given acquisition to symptomatic pepper plants for 48 h followed by inoculation of 45 (three groups of 15) disease-free pepper (cv. Godzilla) plants (10 whiteflies/plant) in clip cages for another 48 h. The inoculated leaves were snapped from the plants after 10 days postinoculation. Whiteflies released to disease-free plants without acquisition access were used as negative controls. Inoculated plants were tested for the presence of the virus by RT-PCR after 20 days. *Rickettsia* infections in MEAM1 populations in Israel have been on the decline, and such infections have not been detected in the last 2 years. Thus, MEAM1 populations free of *Rickettsia* were used in this study.

In another experimental setup, higher numbers (~30 whiteflies/plant) of *B. tabaci* (MEAM1) reared on virus-infected plants were released freely onto 24 disease-free pepper plants for lifelong exposure inside a glass house. Plants were tested for the virus after 20 days postinoculation. An experiment examining PeVYV-2 transmission by *B. tabaci* was conducted by acquisition access of whiteflies (~150) for 48 h on a PeVYV-2-infected pepper leaflet free of aphids, followed by lifelong inoculation access to five virus-free healthy pepper plants. The inoculated pepper plants were tested for PeVYV-2 by RT-PCR using specific primers (Table 1) after 20 days postinoculation.

Localization of PeWBVYV in pepper and whitefly tissues. Localization of PeWBVYV in infected pepper leaves and *B. tabaci* reared on infected plants was done as described previously (34) with Cy3-labeled virus-specific probe (Table 1).

Construction of full-length infectious cDNA clones of PeWBVYV and transmission by whiteflies. The full-length viral genome was assembled from overlapping fragments by overlap extension PCR as described by Shevchuk et al. (35). Briefly, the three fragments showing overlap were PCR amplified separately from cDNA constructed from an infected sample using Q5 High-fidelity DNA polymerase (NEB) and subjected to gel purification, and 120-ng volumes of the fragments were mixed. This mixture of the three fragments was used as the template for first-step overlap PCR in a 50-μl reaction mixture containing 0.6 mM deoxynucleoside triphosphates (dNTPs) and 2% dimethyl sulfoxide (DMSO), with PCR cycling conditions of 98°C for 30 s; 20 cycles of 98°C for 10 s, 50°C for 30 s, and 72°C for 6 min; 72°C for 2 min; and a hold at 4°C. A 2-μl volume of this PCR product was used as the template for 2nd-step PCR with Q5 High-fidelity DNA polymerase in a 50-μl reaction mixture containing 0.6 mM dNTPs and 0.4 μM forward and reverse primers (Table 1) with sequences overlapping with pJL89 binary vector (36). PCR conditions of 98°C for 30 s; 15 cycles of 98°C for 10 s, 60°C for 20 s, and 72°C for 6 min; 20 cycles of 98°C for 10 s. 60°C for 20 s, and 72°C for 7 min; 72°C for 2 min; and a hold at 4°C were used. A 240-ng volume of the gel-purified final PCR product was ligated to 120 ng of PCR-linearized (Table 1) pJL89 binary vector by the use of Gibson assembly mastermix (NEB) for 2 h. A 2-μl volume of the ligation mixture was transformed to Stellar competent cells (TaKaRa) and grown overnight on LB agar with kanamycin. Colonies were screened for full-length virus by colony PCR using primers (Table 1) followed by plasmid extraction and sequencing from positive clones. The sequence of the full-length clones joined by overlap PCR was confirmed by Sanger sequencing. Plasmids were transformed into *Agrobacterium tumefaciens* (GV3101) using a freeze-thaw method. Infectivity of the clones was tested by infiltration into *Nicotiana benthamiana* as described previously (36). Pepper seedlings (10 days old) were inoculated with infectious clones by injection at the shoot tip by the use of a fine needle. Replication of viral copies in *N. benthamiana* and pepper was confirmed by Northern blotting with a digoxigenin (DIG)-labeled 3′-specific probe (37). Noninoculated leaves from the top of the plant were used for extraction of RNA for Northern blot analysis. The availability of virions in the phloem cells of *N. benthamiana* leaves and midguts of *B. tabaci* upon acquisition access to the clone-infected plants was confirmed by FISH under a confocal microscope using a PeWBVYV-specific probe. *B. tabaci* whiteflies (MEAM1; *Rickettsia* free) reared on cotton were released onto an infected pepper plant for acquisition for 48 h and were given inoculation access for 7 days to six noninfected pepper plants to test transmissibility of the infectious

clone, and the setup was replicated once. A control was also set up with *B. tabaci* whiteflies given acquisition and inoculation access to noninfected pepper plants. Total RNA was extracted from the top leaves of the inoculated pepper plants (20 dpi) and was tested for PeWBVYV by RT-PCR.

Virus detection and distribution in Israel. Specific detection of the virus was done by RT-PCR using a newly designed primer set, AeYV-F and AeYV-R (Table 1), and RNA extracted from leaves and fruits of pepper plants. A multiplex PCR assay for simultaneous detection of the recombinant virus described in this study and the previously described PeVYV-2 from Israel was designed. First-strand cDNA was synthesized using primers specific for both viruses (Table 1) followed by PCR amplification of both viruses using a common forward primer (0.5 μM) and two specific reverse primers (0.25 μM each; Table 1) for the respective viruses. PCR conditions of 95°C for 3 min; 35 cycles of 95°C for 20 s, 55°C for 30 s, and 72°C for 1 min; and 72°C for 5 min were used. This multiplex assay was used to survey the distribution of the two viruses in major pepper-growing areas in Israel. Surveys were conducted for two successive cropping seasons (January to April 2017 and 2018). Leaf/fruit samples from symptomatic pepper plants were collected from net houses and greenhouses in the Jordan Valley and Arava Valley and the coastal region and were tested for the presence of both viruses.

Data availability. The sequence determined in this work is available in GenBank under accession no. MK333461.

ACKNOWLEDGMENT

We express our gratitude to Bryce Falk (University of California [UC], Davis) for providing the binary vector (pJL89).

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