Biological and Molecular Properties of *Wild potato mosaic virus* Isolates from Pepino (*Solanum muricatum*)

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Abstract

In 1976, a virus with flexuous, filamentous virions typical of the family *Potyviridae* was isolated from symptomatic pepino (*Solanum muricatum*) plants growing in two valleys in Peru's coastal desert region. In 2014, a virus with similar-shaped virions was isolated from asymptomatic fruits obtained from pepino plants growing in six coastal valleys and a valley in Peru's Andean highlands. Both were identified subsequently as *Wild potato mosaic virus* (WPMV) by serology or high-throughput sequencing (HTS). The symptoms caused by two old and seven new isolates from pepino were examined in indicator plants. Infected solanaceous hosts varied considerably in their sensitivities to infection and individual isolates varied greatly in virulence. All seven new isolates caused quick death of infected *Nicotiana benthamiana* plants and more than half of them killed infected plants of *Physalis floridana* and *S. chancayense*. These three species were the most sensitive to infection. The most virulent isolate was found to be BA because it killed five of eight solanaceous host species whereas CA was the least severe because it only killed *N. benthamiana*. Using HTS, complete genomic sequences of six isolates were obtained, with one isolate (FE) showing

evidence of recombination. The distances between individual WPMV isolates in phylogenetic trees and the geographical distances between their collection sites were found to be unrelated. The individual WPMV isolates displayed nucleotide sequence identities of 80.9–99.8%, whereas the most closely related virus, *Potato virus V* (PVV), was around 75% identical to WPMV. WPMV, PVV, and *Peru tomato virus* formed clusters of similar phylogenetic diversity, and were found to be distinct but related viruses within the overall *Potato virus Y* lineage. WPMV infection seems widespread and of likely economic significance to pepino producers in Peru's coastal valleys. Because it constitutes the fifth virus found infecting pepino and this crop is entirely vegetatively propagated, development of healthy pepino stock programs is advocated.

Keywords: American lineage, disease management, economic significance, etiology, high-throughput sequencing, host sensitivity to infection, isolate virulence, pathogen detection, pathogen diversity, pepino, Peru, phylogenetic analysis, bush fruit, symptomatology, virions, viruses and viroids, widespread infection, *Wild potato mosaic virus*.

Pepino (*Solanum muricatum*) is a perennial bush fruit crop first domesticated in the Andean region of South America well before the era of the Inca empire. It is currently grown at altitudes ranging from close to sea level up to 3,000 m, grows best under warm conditions, is propagated only from cuttings, and produces a sweet fruit which tastes like melon. It is sometimes called “pepino dulce” (dulce = sweet) to distinguish it from cucumber (*Cucumis sativus*) which is called “pepino” in Spain and produces fruit that are not sweet. Due to pepino's sensitivity to chilling, pests, and diseases, its crops are replanted annually. Because there has never been a pepino breeding program in South America, its culture in the Andean region depends entirely on traditional land races. These are grown in different locations and their fruit differ in shape, size, and skin coloration. Recently, pepino became adopted as a crop by several other countries, including New Zealand, Australia, Spain, Turkey, Israel, and China, in some of which pepino breeding is underway to improve

the crop (Blanca et al. 2007; Herraiz et al. 2015, 2016; Prohens et al. 1996).

In the coastal desert region of Peru, rivers that flow from the Andes mountains down to the Pacific Ocean are used to irrigate crops, including pepino, a crop grown mostly by smallholder farmers. In 1974, two viruses were found infecting symptomatic pepino plants growing in irrigated crops in the same coastal desert region. These were the Andean strain of *Potato virus S* (PVS^A; genus *Carlavirus*, family *Betaflexiviridae*) and a new virus named *Pepino mosaic virus* (PepMV; genus *Potexvirus*, family *Alphaflexiviridae*) (Jones et al. 1980). In 1976, a third virus was isolated from pepino plants growing in the same region. It had 750-nm flexuous, filamentous particles typical of the genus *Potyvirus* (family *Potyviridae*) (R. A. C. Jones, unpublished data). In 1986, this potyvirus was reisolated from pepino fruit and identified by inoculation to indicator hosts and serology as *Wild potato mosaic virus* (WPMV) (C. E. Fribourg, unpublished data). WPMV had been found earlier (in 1974) in naturally infected plants of the wild potato species *S. chancayense* showing symptoms of severe mosaic and leaf deformation growing in “mist-watered” natural vegetation known as the “Lomas” in the Peruvian coastal desert region (Dillon 2005; Jones and Fribourg 1979, 1990). In addition, in the 1970s, two other viruses were found infecting pepino crops in the same region, *Cucumber mosaic virus* (CMV; genus *Cucumovirus*, family *Cucumoviridae*) (Fernandez-Northcote et al. 1982), and *Tomato mosaic virus* (ToMV; genus *Tobamovirus*, family *Virgaviridae*) (C. E. Fribourg, unpublished data). Also, another virus, initially called *Pepino latent virus*, was detected infecting pepino cuttings introduced to New Zealand from Chile but was subsequently shown to be PVS^A (Dolby and Jones 1988; Santillan et al. 2018; Thomas et al. 1980). Occurrence of all of these viruses in a crop that is only propagated vegetatively, depends entirely on local

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Funding: Operating funds in Lima were provided by the International Potato Center in 1976 to 1978 and National Agrarian University in 2014 to 2016; in 2016 to 2017 they were provided by the U.K. Government's Department of Environment Food and Rural Affairs (DEFRA) Future Proofing Plant Health Project.

The author(s) declare no conflict of interest.

Accepted for publication 22 February 2019.

land races, and for which no healthy stock program is available, is cause for considerable concern. This is because of the presence of multiple virus infection, potentially leading to major fruit yield losses and economic hardship to the smallholder farmers that depend on the pepino crop for a living.

Also in the 1970s, another potyvirus was described in the same region infecting the solanaceous crops tomato (*S. lycopersicon*) and pepper (*Capsicum baccatum* var. *pendulum*) and named *Peru tomato virus*

(PTV) (Fernandez-Northcote and Fulton 1980; Fribourg 1979; Fribourg and Fernandez-Northcote 1982). Phylogenetic analysis using complete genomic sequences of PTV and WPMV confirmed that both were distinct potyviruses (Spetz et al. 2003). This analysis also revealed that they and another potyvirus first found infecting cultivated potato in the Andean highlands of Peru, *Potato virus V* (PVV) (Fribourg and Nakashima 1984; Jones and Fribourg 1986), were more closely related to each other than to any other potyviruses (Spetz and Valkonen 2003).

Fig. 1. Map of Peru showing the three regions the country is divided into and the collection sites of the 10 *Wild potato mosaic virus* (WPMV) isolates used in this study. The narrow region on the left is the coastal desert, the central longitudinal region is the Andean highlands, and the Amazon jungle region is on the right. Nine WPMV isolates were collected in the coastal region and a single isolate (CH) in the highlands.

In 2014, WPMV was isolated again from asymptomatic pepino fruit harvested from plants growing in smallholder fields at six locations in the central and northern coastal regions and one in the northern Andean highlands of Peru. Because WPMV was found in all pepino fruit tested, infection with this virus was apparently very widespread. This article greatly expands knowledge of the host reactions and phylogenetics of this virus, previously based entirely on studies with the WPMV type strain isolated from *S. chancayense* in 1974 (Jones and Fribourg 1979, 1990) or a subsequent subculture of it (Spetz and Valkonen 2003). It describes the biological properties of the pepino isolates and shows that they vary greatly in virulence and, depending on the isolate and host species, infection is sometimes lethal in several solanaceous host species. Using their open reading frame (ORF) sequences, it also determines the phylogenetic relationships between six WPMV isolates from pepino, the type strain from *S. chancayense*, and the potyviruses PTV and PVV from solanaceous crops in the region.

Materials and Methods

Plants and mechanical inoculations. Indicator hosts were grown in a sterile mix of soil, peat, and sand (1:1:1). All plants were maintained at 18 to 22°C in insect-proof greenhouses located at La Molina, Lima, Peru. The studies in 1976 to 1978 were undertaken in International Potato Center greenhouses, and those with the seven isolates from pepino in 2014 to 2016 1 km away in a National Agrarian University greenhouse. Mechanical inoculations to indicator hosts were made by rubbing cotton swabs dipped in infective leaf sap diluted in distilled water onto leaves previously dusted with 600-mesh-sized carborundum powder. Plants of *Nicotiana debneyi*, *N. glutinosa*, *N. occidentalis*, *N. rustica*, and the cross *N. bigelovii* × *N. clevelandii* (F1 generation) were used to culture the different WPMV isolates.

Virus isolates. In 1976 to 1977, samples were collected from young leaves of pepino plants showing mosaic and mild rugosity symptoms growing in small, irrigated fields at Cieneguilla in the Lima Valley and Puente Negro in the Canete Valley, both in the central coastal region of Peru (Fig. 1). To obtain virus isolates, sap extracts from these leaf samples were inoculated to *N. glutinosa* plants. No single lesions isolations were attempted. The two potyvirus isolates (CI and PN) that resulted (Table 1) remained unidentified until 2015.

In 2014, symptomless pepino fruit from plants growing in smallholder fields were collected at six locations (Canete, Huaral, Barranca, Huarmey, Talambo, and Ferrenafe) in the central and northern coastal regions and one at Choropampa (Jequetepeque Valley) in the northern Andean highlands 150 km from the coast. From each location, fruit mesocarp tissue from two ripe fruit was combined and ground in distilled water, and the extract inoculated to leaves of two *N. occidentalis* plants. Once systemic mosaic symptoms developed, leaf tissue from the plant that developed the severest symptoms

was desiccated over silica gel and stored for subsequent study in 2015 to 2016 (1 isolate/location). The seven isolates obtained in this way were named after each of the locations: CA, HR, BA, HU, TL, FE, and CH (Fig. 1; Table 1). In 2015 to 2016, each of them was reisolated from this desiccated tissue by grinding it in distilled water, followed by inoculation of the sap extract to *N. occidentalis* plants. Then, to eliminate the possibility of mixtures with other viruses, each isolate was passaged through single local lesions in inoculated leaves produced by inoculation of infective *N. occidentalis* sap to *Chenopodium quinoa* plants. Leaf sap extracts from culture hosts infected with all seven isolates gave positive reactions when tested by enzyme-linked immunosorbent assay (ELISA) using WPMV antiserum.

In 1978, isolate SC-, CI-, and PN-infected leaf samples were dried over silica gel in Peru, sealed in glass vials, and sent to the United Kingdom. Isolate SC was the type strain of WPMV isolated from *S. chancayense* (Jones and Fribourg 1979). The tin in which these vials were sent currently forms part of the plant virus collection at Fera Science Ltd., York, U.K. In 2016, leaf material containing each of the seven new WPMV isolates from pepino was also dried over silica gel in Peru before sending to Fera Science Ltd.

Serological tests. Serological tests to detect virus infection in plant samples were undertaken using microprecipitin (1976 to 1978) or ELISA (2014 to 2016) tests. Microprecipitin tests were used routinely for serological detection in Peru in the 1970s (Fribourg et al. 1977a,b; Moreira et al. 1980) but, later, direct double-antibody sandwich ELISA was used instead. The procedures employed for these two kinds of standard serological tests were described previously (Ball 1974; Bercks et al. 1972; Clark and Adams 1977). The polyclonal antisera to WPMV type strain (isolate SC), PVS, and PepMV used in these tests were those used previously in La Molina, Lima, Peru (Jones and Fribourg 1979; Jones et al. 1980; Santillan 1979; Santillan et al. 1980).

Symptomatology. In 1977 to 1978, infective leaf sap from *N. glutinosa* plants infected with isolates CI and PN were inoculated to two plants each of several standard indicator host species frequently used with viruses isolated from solanaceous plants; healthy uninoculated plants of each species were kept as controls. Any virus symptoms that developed were recorded. In 2015 to 2016, the seven recent WPMV isolates were inoculated to healthy plants of (i) the wild potato *S. chancayense*, from which isolate SC originally came (Jones and Fribourg 1979); (ii) five indicator host species previously inoculated with isolates CI and PN; (iii) five other indicator host species previously inoculated with isolate SC; and (iv) the cross *N. bigelovii* × *N. clevelandii*. Leaf sap from infected culture plants of *N. rustica*, *N. occidentalis*, or the cross *N. bigelovii* × *N. clevelandii* was used for these inoculations. Each isolate was inoculated to three plants of each species. Healthy uninoculated plants of each of them were kept as uninfected controls. Any symptoms that developed were

Table 1. Details of Wild potato mosaic virus isolates used in this study

Isolate	Original isolation host host species	Symptoms in original isolation host	Where collected or obtained in Peru ^a	Isolation year	GenBank code	Reference
W ^b	<i>Solanum chancayense</i>	Mosaic, leaf deformation (foliage)	Lachay, Chancay Valley (Lomas)	1974	AJ437279	Spetz and Valkonen (2003)
SC	<i>S. chancayense</i>	Mosaic, leaf deformation (foliage)	Lachay, Chancay Valley (Lomas)	1974	MK092723	Jones and Fribourg (1979)
(Type)						
CI	<i>S. muricatum</i>	Mosaic, rugosity (foliage)	Cieneguilla, Lima Valley	1976	Short sequences	This study
PN	<i>S. muricatum</i>	Mosaic, rugosity (foliage)	Puente Negro, Canete Valley	1977	MK092722	This study
CA	<i>S. muricatum</i>	Symptomless infection (fruit)	Canete, Canete Valley	2014	No sequence	This study
HR	<i>S. muricatum</i>	Symptomless infection (fruit)	Huaral, Chancay Valley	2014	MK092721	This study
BA	<i>S. muricatum</i>	Symptomless infection (fruit)	Barranca, Pativilca Valley	2014	MK092720	This study
HU	<i>S. muricatum</i>	Symptomless infection (fruit)	Huarmey, Huarmey Valley	2014	No sequence	This study
TL	<i>S. muricatum</i>	Symptomless infection (fruit)	Talambo, Jequetepeque Valley	2014	MK092724	This study
FE	<i>S. muricatum</i>	Symptomless infection (fruit)	Ferrenafe, Ferrenafe Valley	2014	MK092725, recombinant	This study
CH	<i>S. muricatum</i>	Symptomless infection (fruit)	Choropampa, Jequetepeque Valley	2014	No sequence	This study

^a Choropampa is located in the Andean highlands of Peru. All the other valleys are located in the country's Pacific coastal region.

^b Renamed subculture of isolate SC sequenced by Spetz and Valkonen (2003).

recorded. Samples from inoculated and tip leaves of all hosts were collected 3 weeks after inoculation and tested for WPMV infection by inoculation to two plants of *N. rustica*.

Host species sensitivity and isolate virulence. All nine virus isolates from pepino killed at least one solanaceous host species to which they were inoculated; therefore, induction of plant death from inoculation was used to estimate both host species sensitivity to infection and isolate virulence. Host species killed by many isolates were considered sensitive and those killed by no or one isolate as tolerant. Isolates that killed many host species were considered virulent, whereas those that killed one or no host species were designated avirulent. The phenotypes that developed in the eight hosts inoculated by all seven new pepino isolates (CA, HR, BA, HU, TL, FE, and CH) were mainly used for this purpose. The phenotypes that developed in the five solanaceous hosts inoculated solely with SC and the two old pepino isolates (CI and PN) were less useful, although some information on relevant differences was deduced from them.

Electron microscopy. In 1977, crude sap preparations from isolate CI- and PN-infected leaves of infected plants were mixed with 2% sodium phosphotungstate (pH 6.5) and sprayed onto carbon-coated film on copper grids, as described by Roberts (1982). The grids were examined for virus particles using a JEM-100S electron microscope (JEOL USA Ltd.).

High-throughput sequencing. In 2015 to 2017, samples of desiccated WPMV-infected leaf material containing seven isolates (SC, CI, PN, HR, BA, TL, and FE) were subjected to high-throughput sequencing (HTS) (Table 1). For this, total RNA was extracted from each sample using the Plant Total RNA kit (Qiagen or Sigma-Aldrich), including the optional DNase treatment. An indexed plant ribosome subtracted sequencing library was then produced from the total RNA using the Scriptseq complete plant leaf kit (Illumina) following the manufacturer's instructions. The indexed library was then sequenced along with others on a MiSeq instrument (Illumina), using a 600-cycle V3 kit. The resulting paired reads were 3' trimmed to a quality score of 20 using Sickle in paired-end mode (Joshi and Fass 2011). Contigs were assembled using Trinity v2 at maximum memory allocation of 99 gigabytes of RAM and 64 central processing units allocated to the process (Grabherr et al. 2011). BLAST+ version 2.2.29 was employed to compare the contigs produced to the GenBank nonredundant and nucleotide databases (Camacho et al. 2009). Reads of viral origin were extracted using the extract reads function in MEGAN Community Edition 6.10.2 (Huson et al. 2016). Isolates SC, PN, HR, BA, TL, and FE provided complete WPMV ORFs but isolate CI only two short sequences. No other virus sequences were associated with the complete ORFs of WPMV isolates PN, HR, BA, TL, and FE. However, a yet-to-be identified partitivirus sequence was present with the complete ORF of isolate SC and a CMV sequence with the two short isolate CI sequences. The six new genomic sequences with complete ORFs were 9,195 nucleotides long, and the two CI sequences were 3,414 and 1,925 nucleotides long. Final nucleotide sequences of the six isolates with complete ORFs were submitted to GenBank with accession numbers MK092720 to MK092725 (Table 1).

Recombination, sequence identities, and phylogenetic analyses. Sequences were edited using BioEdit (Hall 1999). They were aligned using Clustal W in MEGA 7.0.14 (Kumar et al. 2016), MAFFT in Geneious (Katoch and Standley 2013), or the TranslatorX online server using encoded amino acids as a guide and its MAFFT option (Abascal et al. 2010) (<http://translatorx.co.uk>). The six new complete WPMV ORF sequences were aligned with the isolate W (AJ437279) sequence and this alignment was searched for evidence of genetic recombination using the full suite of options in RDP4 with default parameters (Boni et al. 2007; Gibbs et al. 2000; Holmes et al. 1999; Lemey et al. 2009; Martin and Rybicki 2000; Martin et al. 2005, 2015; Maynard Smith 1992; Padidam et al. 1999; McGuire and Wright 2000; Posada and Crandall 2001). Isolate W had been derived from a Scottish subculture of original WPMV type isolate SC, and subsequently renamed isolate W prior to sequencing (Spetz and Valkonen 2003; Spetz et al. 2003). Percentage nucleotide identities between genomes were calculated

using a matrix of nucleotide identities produced using Sequence Demarcation tool v1 (Muhire et al. 2014).

Mega 7 (Kumar et al. 2016) was used to determine the most appropriate replacement models for computing maximum-likelihood (ML) trees of complete nucleotide ORFs and their encoded amino acid sequences. The BlastN and BlastP online facilities of GenBank (Altschul et al. 1990) were used to search for sequences related to WPMV. Phylogenetic trees were calculated using PhyML 3.0 (Guindon and Gascuel 2003). Statistical support for their nodes was calculated by the SH method (Shimodaira and Hasegawa 1999). Trees were drawn using Figtree 1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>) and a commercial computer illustration package.

The ML tree calculated from nucleotide sequences was rooted using the midpoint option in Figtree. It compared the ORFs of the five nonrecombinant WPMV isolates with those of six PVV sequences and two sequences of PTV (synonym *Peru tomato mosaic virus* [PTMV]; AJ437280 and AJ516010). The outgroups consisted of the ORFs of single sequences of *Mashua virus Y* (MH680824) (Adams et al. 2018b), *Brugmansia mosaic virus* (JX874139) (Damsteegt et al. 2013), and *Pepper mottle virus* (EU586121) (Kim et al. 2009). The ML tree calculated from encoded amino acid sequences compared the main ORFs of single representative, mostly complete genomes of 27 members of the *Potato virus Y* (PVY) lineage found in GenBank in October 2018. For this, two WPMV ORFs, those of MK092723 (SC) and MK092722 (PN), were used to search the GenBank database, and the full genomic sequences of closely related potyviruses were identified and downloaded. Their ORFs were aligned with those of all five complete nonrecombinant WPMV isolates. The alignment also included partial sequences of closely related potyviruses, consisting mostly of their Nib plus coat protein sequences. The positions and relative branch lengths of these partial sequences were calculated using only those regions shared by the partial and complete polyprotein sequences.

Results

Isolate purity. In 1977, electron microscopy was used to examine leaf sap extracts from *N. debneyi* and *N. rustica* plants infected with isolates CI and PN, respectively. In both instances, flexuous filamentous virions 750 nm in length typical of potyviruses were present but no other virions were recorded. Microprecipitin tests on leaf sap extracts from the infected *N. glutinosa* plants failed to detect PepMV. Similarly, no evidence of another virus being present was found when sap extracts containing isolate SC were examined in the electron microscope, tested serologically, or inoculated to indicator hosts (Jones and Fribourg 1979). However, when HTS was used with these three isolates, in addition to WPMV sequences, the presence of CMV and partitivirus sequences was revealed with isolates CI and SC, respectively (see above).

All seven new isolates that were passaged through single local lesions provided cultures that gave a positive reaction when extracts of samples from their infected leaves were tested by ELISA using WPMV antiserum. However, they were free from CMV, PepMV, and ToMV for several reasons. Inoculation of indicator hosts with infective *N. occidentalis* sap never caused any phenotypic reactions typical of these viruses in any indicator host. For example, none of them ever induced any local lesions in inoculated leaves of *N. glutinosa* (ToMV indicator host) (Hollings and Huttinga 1976) or *Vigna unguiculata* (CMV indicator host) (Franccki et al. 1979), or any local or systemic symptoms in *Datura stramonium* (PepMV indicator host) (Jones et al. 1980). Also, none of them induced the systemic mottle in *C. quinoa* plants typical of PVS^A (Hinojosa-Orihuela 1973; Santillan et al. 1980, 2018), and HTS did not reveal any admixture with other viruses (see above).

Symptomatology. All isolates from pepino infected the indicator host plants inoculated with them (Table 2) but there was considerable variation in the symptom types and severities they elicited (Table 2; Fig. 2). In contrast, isolate SC failed to infect *C. quinoa* and *N. glutinosa*. In *N. rustica*, all nine pepino isolates resembled isolate SC in inducing chlorotic or necrotic local lesions (Fig. 2A) and systemic

chlorotic or necrotic spots, blotches, or rings (Fig. 2B, left and right) but BA also induced plant death. In *N. tabacum* ‘Samsun’, isolates CI and HU resembled isolate SC in inducing asymptomatic infection in inoculated leaves instead of the local necrotic lesions elicited by the other seven isolates. Systemic necrotic rings were induced in this host

by five pepino isolates and isolate SC (Fig. 2C, left) but not by CI, PN, HR, and HU. In addition, except with isolates SC, CI, and PN, veinal necrosis (Fig. 2C, right) was recorded for all isolates, which extended to the petioles and stems with isolates CA, HR, and HU (Fig. 2D). In the F1 cross *N. bigelovii* × *N. clevelandii*, all seven

Table 2. Symptoms caused by nine *Wild potato mosaic virus* isolates in indicator hosts and *Solanum chancayense*

Host, infection	Isolate ^a									
	SC	CI	PN	CA	HR	BA	HU	TL	FE	CH
<i>Chenopodium quinoa</i>										
Local	NI	NS	NS	CS, NS	CS NS	NS	CS, NS	NS	NS	NS
Systemic	NI	NI	NI	0	0	0	CS, NS	0	0	NS
<i>Nicandra physaloides</i>										
Local	0	...	...	CS	CS	NS	NS	CS	CS	NS
Systemic	M	...	...	VC, CS, UCTL, M	VC, CS, UCTL, M, LDr	VC, CS, NS, LEp, GN, PD	VC, LEp, UCTL, GN, PD	VC, LEp, UCTL	VC, LEp, UCTL	VC, NS, LEp, UCTL
<i>Nicotiana benthamiana</i>										
Local	0	...	...	CS	CS	CS	CS	CS	CS	NS, NR
Systemic	0	...	...	VC, LEp, M, GN, PD	VC, LEp, M, GN, PD	VC, LEp, M, GN, PD	VC, LEp, M, GN, PD	VC, LEp, M, GN, PD	VC, LEp, M, GN, PD	VC, VN, LEp, GN, PD
<i>N. bigelovii</i>										
Local	0	NS	0	...	...	...	...	...	...	...
Systemic	M, LDr	VC, M, LDr, GN, PD	VC LSr-M	...	...	...	...	...	...	...
F1 <i>N. bigelovii</i> × <i>N. clevelandii</i>										
Local	...	...	...	CS	CS	CS, CR	CS	CS, NS	0	CS, CR
Systemic	...	...	...	VC, NS, M, LCr, LSr	VC, M, LSr	VC, M, LCr, LSr	M, LCr	M, LCr	M, LCr	VC, NS, M, LSr
<i>N. clevelandii</i>										
Local	0	NS	NS, NR	...	...	...	...	...	...	...
Systemic	M	NS, M, LDr, LD, GN, PD	VC, NS, LD, GN, PD	...	...	...	...	...	...	...
<i>N. debneyi</i>										
Local	0	0	CS	...	...	...	...	...	...	...
Systemic	0	VC, LSr-M	LSr-M	...	...	...	...	...	...	...
<i>N. glutinosa</i>										
Local	NI	0	0	...	...	...	...	...	...	...
Systemic	NI	VC, LSr-M	VC, LSr-M	...	...	...	...	...	...	...
<i>N. occidentalis</i>										
Local	0	NS	NS	NS	NS	NS	NS	CS	CS	NS
Systemic	VC, M, UCTL	LDr, GN, PD	VC, LSr-M	VC, M	VC, NS, M	VC, NS, M	VC, NS, M, LCr	VC, M	VC, M	VC, NS, M, LCr
<i>N. rustica</i>										
Local	CB, NR	NR	NR	CS	NS	NS	NS, NR	CS	CS	CS
Systemic	CB, 0	VC, CS	VC, CS, 0	VC, CS, CR, LCr, M	VC, CS, NB, LCr	VC, NS, CB, PD	VC, CS, NB	VC, CR, CB, M	VC, CR, CB, M	VC, CS, LCr
<i>N. tabacum</i> ‘Samsun’										
Local	0	0	NS	NS, NR	NS, NR	NS	0	NS, NR	NS, NR	NS, NR
Systemic	NS, NR, CB	M	VC, M	VC, VN, NB, INR, SN	VC, VN, NB, SN	VN, INR	VC, VN, NB, SN	VC, NB, VN INR	VC, VN, NB, INR	VC, VN, NB, INR
<i>Physalis floridana</i>										
Local	0	...	...	0	0	0	0	0	0	0
Systemic	M	...	...	VC, LEp, LDr	VC, LEp, LDr	VC, LEp, GN, PD	VC, LEp, GN, PD	VC, LEp, GN, PD	VC, LEp, GN, PD	VC, LEp, GN, PD
<i>Solanum chancayense</i>										
Local	0	...	...	NS, LCl, LDr	LCl, LDr	NS, LCl, LDr	CS, LCl, LDr	CS, LCl, LDr	CS, LCl, LDr	NS, LCl, LDr
Systemic	M, LDr	...	...	LEp, LCl, LN, LSr-M, LDr	LEp, LSr-M, SN, PD	LCl, LN, SN, PD	LEp, LCl, SN, PD	LEp, LCl, LSr-M, LDr	LEp, LCl, LSr-M, LDr	LEp, LCl, LN, PD

^a Data for isolate SC (= type strain) from Jones and Fribourg (1979); studies undertaken in 1975 to 1978 (SC, CI, and PN) or 2015 to 2016 (other seven isolates). Coded symptom descriptions: CB = chlorotic blotches, CS = chlorotic spots, CR = chlorotic rings, GN = generalized necrosis, INR = interrupted necrotic rings, LCr = leaf crinkling, LCl = leaf chlorosis, LEp = leaf epinasty, LD = leaf death, LDr = leaf deformation, LDr = leaf drop, LN = leaf necrosis, LSr = leaf size reduction, LSr-M = leaf size reduction and mosaic of top leaves, M = mosaic, NB = necrotic blotches, NI = not infected, NR = necrotic rings, NS = necrotic spots, **PD** (in bold) = plant death, SN = stem necrosis, UCTL = upward curling of top leaves, VC = vein clearing, VN = vein necrosis, and 0 = symptomless infection.

new pepino isolates caused mosaic (Fig. 2E) but CA and CH also produced necrotic spots and smaller-sized top leaves. Except for CH, which caused necrotic spots or rings (Fig. 2I, left) and SC, which caused symptomless infection, the remaining six new pepino isolates all induced chlorotic spots in inoculated leaves of *N. benthamiana*. In addition, in contrast to SC, which was asymptomatic, all seven of them caused systemic necrosis (Fig. 2I, right) followed by plant death. In *Physalis floridana*, with the sole exception of SC, which induced mosaic, all seven isolates tested elicited systemic vein clearing and leaf epinasty. Also, apart from SC, CA, and HR, all of them caused systemic necrosis and plant death. Seven isolates caused local necrotic spots in *N. occidentalis* (Fig. 2F) but, with TL and FE, the lesions were only chlorotic and, with SC, infection was asymptomatic in inoculated leaves. In addition,

apart from CI, all isolates caused systemic vein clearing but HR, BA, HU, and CH also induced systemic necrotic spots (Fig. 2G, left) which spread irregularly, causing leaf crinkling in plants infected with HU and CH (Fig. 2G, right). All seven new pepino isolates induced systemic vein clearing in *Nicandra physaloides* but varied in the other types of symptoms they elicited. For example, SC, CA, and HR failed to induce epinasty of middle leaves, only SC and BA failed to induce characteristic upward curling of top leaves (Fig. 2J), and only BA and HU caused plant death; SC only induced mosaic. Except with SC, which failed to infect *C. quinoa*, all isolates induced local chlorotic or necrotic spots in this species (Fig. 2H), and all seven new pepino isolates caused systemic infection that was mostly asymptomatic; however, HU and CH induced systemic necrotic spotting. When the additional species *S.*

Fig. 2. Symptoms produced in leaves and plants of indicator hosts caused by infection with different *Wild potato mosaic virus* isolates. **A**, Left to right: Local chlorotic spots, local chlorotic spots surrounded with necrotic rings, and local necrotic spots in inoculated leaves of *Nicotiana rustica* caused by infection with isolates CH, HU, and BA, respectively. **B**, Left to right: Systemic chlorotic rings and systemic necrotic spots surrounded by chlorotic areas in *N. rustica* caused by infection with isolates TL and BA, respectively. **C**, Left to right: Systemic necrotic interrupted rings and systemic vein necrosis in *N. tabacum* 'Samsun' caused by infection with isolates CA and HR, respectively. **D**, Systemic vein, petiole, and stem necrosis in *N. tabacum* 'Samsun' caused by infection with isolate HU. **E**, Systemic mosaic in F1 cross of *N. bigelovii* × *N. clevelandii* caused by infection with isolate TL. **F**, Irregular local necrotic spots in inoculated leaves of *N. occidentalis* caused by infection with isolate CA. **G**, Left to right: Systemic necrotic spots and leaf crinkling in *N. occidentalis* caused by infection with isolate CH. **H**, Local chlorotic spots in inoculated leaves of *Chenopodium quinoa* caused by infection with isolate HU. **I**, Left to right: Local necrotic rings in inoculated leaves and systemic vein necrosis in *N. benthamiana* caused by infection with isolate CH. **J**, Systemic necrotic spots, leaf epinasty of middle leaves, and upward curling of top leaves in *Nicandra physaloides* caused by infection with isolate CH. **K**, Reduced leaf size, mosaic, and deformation of top leaves in *Solanum chancayense* caused by infection with isolate TL (left, healthy plant).

pimpinellifolium was inoculated with isolates SC, CI, and PN, SC failed to infect it, a systemic mosaic developed with CI, and PN caused local necrotic ring and spot lesions without systemic invasion.

Isolate SC and the seven new pepino isolates all invaded *S. chancayense* systemically. SC induced asymptomatic infection in inoculated leaves whereas the new isolates all induced chlorosis and leaf drop, which was accompanied by chlorotic or necrotic local lesions, with all except isolate HR. All of these isolates except BA, HU, and CH induced a systemic mosaic; all except SC and BA caused leaf epinasty; and all except SC and HR caused leaf chlorosis of middle leaves. Reduction in leaf size and mosaic developed with CA, HR, TL, and FE (Fig. 2K, right) and, with HR, BA, HU, and CH, systemic infection caused plant death.

With isolate CI, there was no evidence of CMV contamination among the phenotypic responses obtained with this isolate (Table 2). Possibly the CI culture became contaminated after the host inoculations were done. Alternatively, the CMV isolate present might have come from mixed infection in the original pepino sample that CI came from but was asymptomatic in the hosts inoculated. Similarly, from the phenotypic responses obtained, there was no evidence of partitivirus contamination in the SC culture. This is expected because partitivirus infection rarely causes disease symptoms in plants, apparently also including when mixed infection occurs with other viruses (Roossinck 2010).

Isolate virulence. When induction of plant death in hosts was used as a measure of isolate virulence, the most virulent isolate was BA because it caused plant death in five of eight solanaceous hosts inoculated with it. HU killed four of eight; thus, it was also relatively virulent. The mildest isolate was SC because it failed to kill any of these solanaceous hosts or the four others it was inoculated to. CA was also mild because it only killed one of eight solanaceous hosts. Isolates HR, TL, FE, and CH were of intermediate virulence because each killed two to three of the eight solanaceous hosts inoculated with them. Isolates CI and PN killed one or three of the six solanaceous hosts they were inoculated with, respectively; thus, CI seemed relatively virulent and PN of relatively low virulence.

Host species sensitivity. Using plant death as a measure of host species sensitivity to infection, *Nicotiana benthamiana* was highly

sensitive because it was killed by all seven new pepino isolates (Table 2). SC was the only isolate that failed to kill it, its systemic infection being asymptomatic. *P. floridana* and *S. chancayense* were also sensitive because they were each killed by four to five of these seven isolates. In contrast, none of the 10 isolates killed *C. quinoa* or *N. tabacum*; thus, these were the most tolerant hosts. *N. rustica* and *N. occidentalis* also seemed tolerant because only 1 each of the 10 isolates killed them. *Nicandra physaloides* was killed by two of the new isolates. Among the four solanaceous hosts only inoculated with SC and the two old pepino isolates (CI and PN), none of the isolates killed *Nicotiana debneyi* or *N. glutinosa*. However, although none of the seven new isolates killed the F1 generation of the *N. bigelovii* × *N. clevelandii* cross, with its parents, both CI and PN killed *N. clevelandii* and CI killed *N. bigelovii*.

Recombination. The ORF of MK092725 (FE) was identified as likely recombinant having as its major parent MK092722 (PN) and minor parent uncertain, perhaps MK092724 (TL), between nucleotides 5,434 and 6,571 and detected by six methods, with probabilities ranging from 6×10^{-3} to 7×10^{-9} . The other recombinant identified was PVV isolate KC433411 (KER.LAL.P) (Shamsadden-Saeed et al. 2014), a definitive recombinant between two PVV isolates; its major parent AJ243766 and minor parent KT985458 between nucleotides 160 and 1,105 were detected by six methods with probabilities ranging from 6×10^{-3} to 8×10^{-16} . The two recombinants were removed from the subsequent phylogenetic analyses.

Sequence identities. In pairwise comparisons, the complete ORF sequences of the six new complete WPMV genomes (SC, PN, HR, BA, TL, and FE) were compared with that of previously sequenced isolate W (Table 1). They had nucleotide identities of 80.9 to 99.8%, whereas the most closely related virus, PVV, had ORFs around 75% identical. The WPMV type isolate SC (MK092723) and isolate W (AJ437279) differed by only 18 nucleotides over their entire 9,195-nucleotide lengths.

Phylogenetic analysis. The WPMV and PVV isolates fell into two statistically separate clusters (Fig. 3). PTV and PTMV formed a similar third cluster and appeared as two isolates of a single virus. Except within the PVV cluster, all nodes had >0.92 SH support. There was no obvious relationship between the phylogenetic distance between individual WPMV isolates (Fig. 3) and the geographical distances between their collection sites (Fig. 1). For example, the type isolate SC was the most phylogenetically distinct from all the others, apart from W. Like three of the four other nonrecombinant WPMV sequences, it came from an isolate collected in central coastal Peru. However, the sequence which was closest to it in the tree (TL) was of an isolate from north coastal Peru. When the two regions of the incomplete CI isolate genome were aligned separately from the others and ML trees were calculated, both placed the CI sequence between those of the PN and TL isolates.

An ML taxonomy calculated from encoded amino acid sequences in which all nodes marked with a cross had >0.90 SH support is shown in Figure 4. The PVY lineage had two main sublineages, the most speciose of which included PVY itself and some viruses that infect nonsolanaceous plants, notably members of the *Compositae* family. The lesser sublineage included WPMV, PVV, PTV, and six others all originally isolated from solanaceous plants in the Americas. Within this sublineage, *Ecuadorian rocoto virus* grouped with WPMV, PVV, and PTV. *Pepper mottle virus*, *Brugmansia mosaic virus*, and *Brugmansia suaveolens virus* formed a second group and *Pepper yellow mosaic virus* and *Tobacco mosquado virus* a third. All except 2 of the 27 viruses of the overall PVY lineage were originally isolated in the Americas.

Discussion

For more than 40 years, WPMV was considered a wild plant virus of no economic significance. However, our research suggests this assumption is likely to be incorrect. Here, we demonstrate for the first time that WPMV not only infects wild potato plants growing in Peru's coastal desert region but also is able to infect irrigated solanaceous crop plants commonly planted there, in this case the vegetatively propagated fruit crop pepino. Presumably, this virus

Fig. 3. Maximum-likelihood phylogeny of the nonrecombinant open reading frame (ORF) sequences of six isolates of *Wild potato mosaic virus* (WPMV), together with those of six of *Potato virus V* (PVV) and two of *Peru tomato virus* (PTV; synonym *Peru tomato mosaic virus*) with three outgroup sequences. Nodes marked with a cross had >0.90 SH support. The outgroup consisted of the ORFs of single sequences of *Mashua virus Y* (MashVY; MH680824), *Brugmansia mosaic virus* (BrugMV; JX874139), and *Pepper mottle virus* (PepMtV; EU586121). Midpoint root calculated by Figtree. The same root was found using the amino acid sequences of the same sequences.

evolved first in the Lomas vegetation and spread later to the pepino crop after its initial domestication in the Andean region and its subsequent planting in irrigated coastal desert fields in close proximity to natural vegetation. In 2014, it was isolated from all pepino fruit samples collected from widely dispersed locations in Peru, suggesting that infection occurs commonly in this crop in different parts of the country. Also, although the original type isolate of this virus from *S. chancayense* mostly caused mild symptoms in standard solanaceous indicator hosts, this was not necessarily so for the WPMV isolates from pepino. Indeed, although infection with the type isolate was never lethal in any host tested, all pepino isolates inoculated to *N. benthamiana* plants killed this species and more than half of them killed plants of *P. floridana* and the wild potato

species *S. chancayense*. Moreover, the individual WPMV isolates tested varied greatly in virulence, and the solanaceous hosts tested varied considerably in their sensitivity to infection. Because the pepino crop is already known to become infected with at least four other viruses in coastal Peru, depends entirely on local land races, and is vegetatively propagated in the complete absence of any healthy stock program, the finding of apparently widespread infection with a fifth virus (WPMV) suggests the likelihood of considerable virus disease-induced losses in its fruit production due to multiple virus infection. In addition, including the six new WPMV genomes obtained in phylogenetic analyses revealed no evidence of any relationship between the phylogenetic distance between individual WPMV isolates and the geographical distances between their original

Fig. 4. Maximum-likelihood phylogeny of 27 *Potato virus Y* (PVY)-lineage viruses with five outgroup viruses calculated from the amino acid sequences of the polyproteins encoded by the open reading frames (ORFs) from single representative, mostly complete genomes of each lineage found in GenBank. To create this, the *Wild potato mosaic virus* ORFs AJ437279 (W) and MK092722 (PN) were used to search the GenBank database, the full genomic sequences of closely related potyviruses were identified and downloaded, and all were aligned. Genomes with black branches were from complete sequences, and those on lighter branches were from partial sequences, mostly their NIb plus coat protein sequence. Positions and relative branch lengths of the partial sequences were calculated using only those regions shared by the partial and complete polyprotein sequences. Nodes marked with a cross had >0.90 SH support. Dark circles mark the viruses reported from the Americas and lighter circles mark those from other regions of the world. Viruses grouped under section A are PVY-lineage viruses, those under section B are the most closely related "other potyviruses", those under section C are representative "basal potyviruses", and section D is a rymovirus (Gibbs and Gibbs 2018).

collection sites. Moreover, only one of them showed evidence of recombination, and WPMV, PVV, and PTV were confirmed to be three distinct but related viruses within the PVY lineage, which is American in origin; therefore, all three apparently evolved in this part of the world.

Further studies are required to provide an improved understanding of WPMV's importance to the pepino crop and the smallholder farmers that grow it. First, a comprehensive survey is required to establish its incidence in Peruvian pepino plantings. Second, WPMV isolates from pepino need to be inoculated to healthy pepino plants to establish their effects on plant growth and fruit yields both alone and in mixed infection with other viruses that infect the crop (currently CMV, PepMV, ToMV, and PVS). In mixed-virus infections potyviruses often stimulate synergistic interactions that greatly increase disease severity (Redinbaugh and Stewart 2018; Untiveros et al. 2007); therefore, study of WPMV's impact in mixed infection would be particularly important here. In addition, inoculation of the WPMV type strain to pepino plants to study its effects on this host would help provide understanding of whether such infection is important for the crop in the presence or absence of other viruses. However, studies on plant growth and fruit yield losses await the development of a healthy stock program to provide virus-tested planting material or the availability of viable botanical seed collected from healthy pepino plants. One WPMV isolate (PN) that came from a diseased pepino plant, which was not passed through single lesions and for which HTS detected no other virus in its culture, was associated with leaf mosaic and deformation symptoms in pepino plants. This is the only information currently available on the effects of single infection with WPMV upon the crop, and there is no information about the effects of mixed-virus infection.

The lethal response to infection exhibited by some combinations of WPMV isolate and solanaceous host species provided a convenient way of establishing the relative virulence of individual isolates and the sensitivities of different host species to infection. Isolate BA seemed highly virulent because it killed the most solanaceous host plant species inoculated by any new pepino isolate. Isolates CA and SC were the least virulent isolates because they killed none (SC) or only one (CA) of the hosts they were inoculated to. *N. benthamiana*, *P. floridana*, and *S. chancayense* were the most sensitive species because, depending upon the relative virulence of the infecting isolate, they developed the most severe symptoms, often including plant death. *N. tabacum*, *N. rustica*, *N. occidentalis*, and the *N. bigelovii* × *N. clevelandii* cross were the most tolerant hosts. This is because, in general, they developed the mildest symptoms even with virulent isolates, being killed by such isolates in either no or only one combination of isolate and host species. A lethal systemic necrotic phenotype is typical of strain-specific systemic hypersensitive response to infection involving single-gene resistance (Jones and Vincent 2018; Nyalugwe et al. 2012, 2016a,b). Whether this is so for WPMV needs to be investigated further but, given that *S. chancayense*, *P. floridana* (= *pubescens*), and *Nicandra physaloides* are wild plants native to the Andean region, including Peru (Brako and Zarucchi 1993), it constitutes a possible explanation for the plant death caused by some isolates in these hosts. Moreover, wild species of potato and tomato from the Andean region have long been known to provide rich sources of virus resistance genes for use in plant breeding, which suggests that such genes may also be present in other wild solanaceous species (Agrama and Scott 2006; Jones 1981; Roselló et al. 1998; Solomon-Blackburn and Barker 2001; Valkonen 1994). However, this explanation seems unlikely to be correct for the species that developed a lethal necrosis phenotype with most WPMV isolates, *Nicotiana benthamiana*, because this is native to the Australian continent rather than to the Andean region of South America where WPMV apparently evolved.

WPMV, PVV, PTV, and PVY are biologically and phylogenetically distinct but related viruses, occur in the same region, and infect related hosts. Therefore, it is interesting that these four viruses form clusters in ML phylogenies of their ORFs with closely similar phylogenetic diversity (PD), which can be estimated as the mean

pairwise patristic distances of all tips (i.e., sequences) connected through the basal node of each cluster (Fourment and Gibbs 2006). This was done for ML phylogenies of the same alignments used for Figures 3 and 4 after adding or replacing representative sequences of the PVY-O phylogroup (AB711147, HQ912877, and KP691317) and PVY-N phylogroup (KX531041, KY847985, and MH795866) (Gibbs et al. 2017). In both phylogenies, the relative PDs of the four viruses were closely similar; that of the WPMV cluster was only 5 to 6% smaller than that of the PVV cluster, only 2 to 3% smaller than that of the PTV cluster, and 10% larger than that of the PVY cluster.

WPMV, PVV, PTV, and six other viruses originally isolated from solanaceous plants all belonged to the smaller of the two sublineages within the overall PVY lineage. All except 2 of the 27 viruses of the PVY lineage were isolated in the Americas, and 17 of them have never been found anywhere else. Moreover, the two not reported first from the Americas still have links with this part of the world. The first, *Amazon lily mosaic virus*, was isolated from *Eucharis grandiflora*, a native of Colombia and Ecuador, and was being grown for its flowers in Taiwan (Fuji et al. 2004). The second, *Amaranthus leaf mottle virus*, was isolated from Argentina amaranth (*Amaranthus diflexus*) growing in Italy (Lovisolo and Lisa 1976; Segundo et al. 2007). This strong association of the PVY lineage with the Americas suggests that it diversified there. However, similar inferences about the basal monocotyledon-infecting viruses of the genus *Potyvirus* suggest that it originated in Western Europe or northwest Africa (Gibbs and Ohshima 2010). If so, an interesting question is how and when did the original ancestor of the PVY lineage migrate from the Old World to the New? More intensive sampling of the populations of the most basal potyviruses in lineages A to D (Fig. 4) (e.g., *Arracacha virus Y*; synonym *Arracacha mottle virus*) (Adams et al. 2018a) in A would be required to resolve this question.

Currently, all we know about WPMV's epidemiology in pepino crops is that its spread is likely to occur through nonpersistent transmission by aphid vectors such as *Myzus persicae* (Jones and Fribourg 1979) and planting infected pepino cuttings. However, movement of infected pepino cuttings between Peru's coastal valleys would explain the lack of any relationship between the phylogenetic distance between WPMV isolates and geographical distance between their collection sites. Although potato and pepper were not infected, tomato and tobacco were experimental hosts of the WPMV type strain (Jones and Fribourg 1979) and tobacco became infected with isolates from pepino in this study. When inoculated in the greenhouse, the wild tomato species *S. pimpinellifolium* became infected with two WPMV isolates from pepino, and the latter species, along with two other wild tomato species (*S. chilense* and *S. peruvianum*), became infected with another pepino virus (PepMV) in the Peruvian coastal desert region (López et al. 2005; Moreno-Pérez et al. 2014). The possible role of tomato, tobacco, wild tomato and wild potato species, and solanaceous agricultural weed hosts such as *P. floridana* (= *pubescens*) and *Nicandra physaloides* in acting as reservoirs of infection for WPMV spread to pepino crops needs to be investigated in the future. The same applies to local climatic drivers of WPMV epidemics in pepino crops. Spread of the virus within the Lomas vegetation also requires attention, including establishing which wild solanaceous species other than *S. chancayense* become infected there, if it poses any threat to the natural ecosystem, and whether the virus spreads readily from this natural vegetation to pepino crops. Regarding WPMV disease management in crops, making healthy pepino planting stock available for use by smallholder farmers seems a logical place to start.

PepMV provides a classic example of a virus formerly localized to one continent being dispersed to the rest of the world via the international seed crop trade, resulting in a serious disease of tomato crops in many continents (Hanssen and Thomma 2010; Jones 2009). Now that we know that WPMV constitutes a potential threat to some solanaceous crops, its seed transmission needs to be investigated as a potential pathway for WPMV dispersion outside South America. Distribution of virus-infected pepino cuttings from South America to another part of the world has already been reported (Dolby and Jones 1988; Thomas et al. 1980). Therefore, importing pepino

cuttings should be avoided unless rigorous postentry quarantine testing for viruses and procedures for their elimination are in place in the importing country.

Acknowledgments

The biological component of this research was undertaken using the glasshouse and laboratory facilities of the International Potato Center (CIP) in 1976 to 1978 and the National Agrarian University in 2014 to 2016, both at La Molina, Lima, Peru. The sequencing component of this research was done using the laboratory facilities of Fera Ltd., Sand Hutton, York, U.K. R. A. C. Jones was an employee of CIP in 1973 to 1978.

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