

Ecological and Genetic Determinants of *Pepino Mosaic Virus* Emergence

Manuel G. Moreno-Pérez,^a Israel Pagán,^a Liliana Aragón-Caballero,^b Fátima Cáceres,^c Aurora Fraile,^a Fernando García-Arenal^a

Centro de Biotecnología y Genómica de Plantas UPM-INIA and E.T.S.I. Agrónomos, Universidad Politécnica de Madrid, Madrid, Spain^a; Facultad de Agronomía, Universidad Nacional Agraria La Molina, Lima, Peru^b; Facultad de Ciencias Biológicas y Agropecuarias, Universidad Nacional de San Agustín, Arequipa, Peru^c

ABSTRACT

Virus emergence is a complex phenomenon, which generally involves spread to a new host from a wild host, followed by adaptation to the new host. Although viruses account for the largest fraction of emerging crop pathogens, knowledge about their emergence is incomplete. We address here the question of whether *Pepino mosaic virus* (PepMV) emergence as a major tomato pathogen worldwide could have involved spread from wild to cultivated plant species and host adaptation. For this, we surveyed natural populations of wild tomatoes in southern Peru for PepMV infection. PepMV incidence, genetic variation, population structure, and accumulation in various hosts were analyzed. PepMV incidence in wild tomatoes was high, and a strain not yet reported in domestic tomato was characterized. This strain had a wide host range within the *Solanaceae*, multiplying efficiently in most assayed *Solanum* species and being adapted to wild tomato hosts. Conversely, PepMV isolates from tomato crops showed evidence of adaptation to domestic tomato, possibly traded against adaptation to wild tomatoes. Phylogenetic reconstructions indicated that the most probable ancestral sequence came from a wild *Solanum* species. A high incidence of PepMV in wild tomato relatives would favor virus spread to crops and its efficient multiplication in different *Solanum* species, including tomato, allowing its establishment as an epidemic pathogen. Later, adaptation to tomato, traded off against adaptation to other *Solanum* species, would isolate tomato populations from those in other hosts.

IMPORTANCE

Virus emergence is a complex phenomenon involving multiple ecological and genetic factors and is considered to involve three phases: virus encounter with the new host, virus adaptation to the new host, and changes in the epidemiological dynamics. We analyze here if this was the case in the recent emergence of *Pepino mosaic virus* (PepMV) in tomato crops worldwide. We characterized a new strain of PepMV infecting wild tomato populations in Peru. Comparison of this strain with PepMV isolates from tomato crops, plus phylogenetic reconstructions, supports a scenario in which PepMV would have spread to crops from wild tomato relatives, followed by adaptation to the new host and eventually leading to population isolation. Our data, which derive from the analysis of field isolates rather than from experimental evolution approaches, significantly contribute to understanding of plant virus emergence, which is necessary for its anticipation and prevention.

Emergent diseases often have a high socioeconomic impact. As described by Woolhouse and Dye (1), an emergent disease can be defined as that “whose incidence is increasing following its first introduction into a new host population, or whose incidence is increasing in an existing host population as a result of long-term changes in its underlying epidemiology.” Viruses account for the largest fraction of emerging diseases in humans, animals, and plants (2, 3). Virus emergence is a complex phenomenon involving multiple ecological and genetic factors, which act during three different phases: in the first phase the virus encounters the new host, often by spread from a wild host; in the second phase the virus adapts to the new host, which involves genetic changes; and in the third phase epidemiological dynamics adapt to the new environment, most often by increasing the between-host transmission rates (3, 4). It is necessary to understand these processes to anticipate and prevent virus emergence. However, understanding plant virus emergence in crops may be limited by a lack of knowledge on the occurrence of virus species and strains in wild plant species from which cross-species spread to crops might occur and on the potential for adaptation to crops of wild-plant-infecting viruses (5, 6).

Pepino mosaic virus (PepMV) (genus *Potexvirus*) is an important pathogen of tomato crops worldwide and a typical example of

an emergent plant virus (6, 7). PepMV has flexuous rod-like particles, which encapsidate a messenger-sense, single-stranded RNA (ssRNA) genome of about 6.4 kb. The PepMV genome encodes five proteins: a protein involved in virus replication (RdRp); three proteins involved in cell-to-cell movement, whose overlapping genes are organized into a triple gene block (proteins TGBp1, TGBp2, and TGBp3); and the coat protein (CP) (8–10). PepMV is transmitted by plant-to-plant contact at high rates, and it is also transmitted through the seed (11, 12), which may have been relevant in its long-distance dispersal.

PepMV was first isolated in 1974 in Peru from pepino (*Solanum muricatum* Ait.) plants showing symptoms of yellow mosaic (10). It was not reported as a pathogen of tomato (*Solanum lycopersicon* L.) (10).

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Address correspondence to Fernando García-Arenal, fernando.garciaarenal@upm.es.

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TABLE 1 Description of wild tomato populations

Population	Altitude, m	Latitude/longitude	Habitat	PepMV incidence, % ^a
Yura 1 (YUR1)	2,636	16°17'S/71°39'W	Mountain short grass	50.0 (3/6)
Yura 2 (YUR2)	2,423	16°14'S/71°42'W	Mountain short grass	0 (0/5)
Cerro Verde 1 (CVD1)	2,236	16°28'S/71°37'W	Mountain short grass	0 (0/5)
Cerro Verde 2 (CVD2)	2,115	16°27'S/71°42'W	Mountain short grass	16.7 (1/6)
Chiguata (CHI1)	2,757	16°25'S/71°27'W	Mountain short grass	31.2 (5/16)
Chiguata (CHI2)	2,847	16°25'S/71°27'W	Mountain short grass	45.5 (5/11)
Mollendo (MOL1)	601	16°57'S/72°3'W	Coastal desert	33.3 (2/6)
Mollendo (MOL2)	544	16°57'S/72°3'W	Coastal desert	25.0 (2/8)
Torata (TOR)	2,170	17°6'S/70°52'W	Mountain short grass	25.0 (4/16)
Samegua (SAM1)	1,653	17°10'S/70°52'W	Coastal desert	0 (0/6)
Samegua (SAM2)	1,712	17°10'S/70°52'W	Coastal desert	50.0 (3/6)
Cerro el Baul (CEB)	1,992	17°7'S/70°50'W	Coastal desert	23.1 (3/13)
Avg				26.9 (28/104)

^a Percentage of infected plants. The number of infected plants/total number of sampled plants are shown in parentheses.

persicum L.) until 1999, in greenhouses in The Netherlands (13, 14). Shortly after, it became a major pathogen of tomato worldwide, with its epidemic expansion being reported first in Europe and then in North America (6–9, 15–18). Isolates from the initial epidemic in Europe were highly similar (99%) to each other, constituting the European tomato strain (EU) (10). EU isolates are closely related (96% similarity) to the strain named Peruvian or LP (7, 10), which includes the original pepino isolate (SM.74) (10) and an isolate from wild *Solanum peruvianum* L. in Peru (19). Since 2005, new PepMV isolates sharing only 80% sequence similarity with the EU or LP strains have been characterized. These isolates were reported from the United States (US1 and US2) (18) and from tomato seeds produced in Chile (CH1 and CH2) (17). Full characterization of these isolates showed that US2 was a recombinant between US1 and CH1, and currently four PepMV strains are recognized (20): the original Peruvian isolate (LP), the European tomato strain (EU), and the American (US1) and Chilean (CH2) strains. PepMV epidemics have been characterized by the replacement of strains. Thus, in Europe EU isolates have been replaced by CH2 isolates (6, 20, 21), with mixed infection between both strains and recombination among them playing a role in the virus evolutionary dynamics (6, 10, 20–22). The US1 genotype has also been found in the Canary Islands (23), and finally, in North America the EU strain became prevalent (24, 25), to be later replaced by the CH2 strain (26).

Characterized isolates from the LP strain are asymptomatic in tomato, and in this host they accumulate to lower levels than the related EU isolates (13, 27, 28), which suggests that emergence of the EU strain could be due to spread of LP isolates to tomato from pepino or wild *Solanum* species, followed by adaptation to tomato. Also, the origin of isolates reported in the United States from tomato seed produced in Chile (CH1 and CH2) (15) is again suggestive of an origin in spread from wild *Solanum* in the areas of seed production. Since the initial emergence of PepMV in tomato crops has been followed by the emergence of new strains causing similar or different symptoms (6, 7, 29), it seems that there is a continued risk of across-host spread and adaptation of new PepMV strains resulting in new emergences.

To test the hypothesis that emergence of PepMV could be due to spread from wild hosts followed by adaptation to tomato, we made a survey for PepMV infection in natural populations of wild tomatoes in southern Peru and analyzed its genetic variation and

population structure. We report the occurrence in wild tomatoes of a new PepMV strain, not yet reported in domestic tomato, and characterized this strain for the traits that may be relevant to evaluate its potential for emergence in tomato crops.

MATERIALS AND METHODS

Field surveys, plant collections, and PepMV detection. Plants of several species of wild tomatoes [*Solanum chilense* (Dunal) Reiche, *S. peruvianum*, *Solanum pimpinellifolium* L., and *Solanum pinmatifidum* Ruiz & Pav.] were sampled in March 2008 at different sites over the distribution of these species in the provinces of Arequipa and Moquegua in southern Peru (Table 1). A total of 12 populations were sampled in different habitats. Relevant information on these populations appears in Table 1. At the time of sampling, plants of all populations were at a similar phenological stage, at flowering but before fruit set. Plants were sampled so that one plant out of every x plants was sampled along fixed itineraries, with itinerary length and x ($0 < x \leq 4$) depending on the population size. For populations consisting of fewer than 10 plants, all individuals were sampled. Samples consisted of 1 to 3 young branches with fresh leaves per plant. Samples were taken to the laboratory, and infection by PepMV was assessed by two complementary procedures: (i) double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using PepMV-specific commercial antibodies (Loewe, Germany) and (ii) reverse transcription-PCR (RT-PCR) using primers PepMVCP-F and PepMVCP-R (sequences are available upon request), which amplified the CP gene.

Nucleotide sequence determination. For all PepMV-positive field plants, RT-PCR amplification of the coat protein (CP) gene was attempted. For this, total RNA extracts were prepared from leaves of field-infected samples using the TRIzol reagent (Life Technologies, CA, USA) following the manufacturer's instructions, and primers PepMVCP-F and PepMVCP-R were used.

The nucleotide sequence of the genomic RNA was determined after transfer of PepMV isolates from a subset of PepMV-positive samples to tomato plants and multiplication. For this, 15-day-old plants of tomato (*S. lycopersicum* cv. Rutgers) were inoculated at the cotyledons with sap of field-infected plants in 0.01 M sodium phosphate buffer, pH 7.0. Inoculated plants were maintained in a growth chamber at 25 or 15°C (for day and night, respectively) and with 15 h of light. The infection status of these plants was checked at 30 days postinoculation (dpi) by DAS-ELISA as described above. To minimize the effect of isolate passage in a different host, leaves from all tomato plants infected with the same isolate were pooled, virus particles were purified as described by Aguilar et al. (8) and disrupted in 4% sodium dodecyl sulfate–0.1 M Tris-HCl (pH 9.0)–1 mg/ml bentonite, and genomic RNA was extracted as described by Pagán et al. (10). The complete nucleotide sequence of the genomic RNA was

obtained for three PepMV isolates. To do so, the sequence at the 5' untranslated region (UTR) was determined using the degenerate primer UTR5'RSL and rapid amplification of cDNA ends (RACE) adapters. The 3' UTR sequence was determined using oligo(dT) and UTR3'FSL. For the rest of the genome, 8 pairs of degenerate primers were used for RT-PCR amplification (Pep1F-Pep1R to Pep8F-Pep8R; sequences are available upon request). Sequences were confirmed on amplicons obtained using specific primers designed on previously sequenced regions (not shown). All amplifications were carried out using the high-yield reverse transcriptase SuperScript III (Life Technologies) and PCR SuperMix high-fidelity polymerase (Life Technologies).

Analyses of PepMV nucleotide genomic sequences. Sequence alignments were constructed using MUSCLE 3.7 (30) and adjusted manually using Se-Al (31). Genetic diversities were estimated using the general time-reversible substitution model with invariant sites and a gamma distribution of among-site rate variation ($GTR+I+\Gamma_4$) as implemented in MEGA 5 (32). This was the best-fitted nucleotide substitution model selected by the corrected Akaike information criterion as determined by jModelTest 0.1.1 (33). Standard errors of each genetic diversity measure were based on 1,000 replicates bootstrap using MEGA 5. Alternative nucleotide substitution models (Kimura 2-parameter model and Tamura-Nei model) yielded similar results. For phylogenetic analyses, the occurrence of recombination in the utilized sequences was determined using four methods based on different assumptions (34), and implemented in RDP3 (<http://darwin.uvigo.es/rdp/rdp.html>) (RDP, BOOTSCAN/RECSCAN, Siscan, and Chimaera, with default parameters) (35). Only recombination signals detected by all methods ($P < 0.05$) were considered positive. Maximum-likelihood (ML) phylogenetic trees were inferred with PhyML (36), using the subtree pruning regrafting (SPR) method and incorporating the $GTR+I+\Gamma_4$ substitution model.

For reconstruction of the ancestral state of host species and PepMV strains and the time scale of virus evolution, the presence of temporal and state-related phylogenetic signals in the analyzed data set is essential to an accurate estimation. Thus, we first assessed the strength of the temporal signal in a data set with 34 complete genomic PepMV sequences. To do so, we constructed an ML tree as described above, and clock-like behavior of the data set was then assessed by regressing the root-to-tip distance in the ML tree against the date of sampling of each sequence using Path-O-Gen v1.4 (<http://tree.bio.ed.ac.uk/software/pathogen/>). To confirm the presence of a temporal signal, the BEAST analyses described below were repeated on data sets in which sampling times were randomized so that the temporal structure was disrupted. Randomizations were repeated 10 times, and substitution rates for randomized and real data were compared. Randomized and real values were considered significantly different when the 95% highest posterior density (HPD) values for all of the randomized controls excluded the mean substitution rates estimated for the real data, indicating the presence of a temporal signal. Second, we analyzed the presence of a phylogenetic signal by calculating the number of steps required for parsimony reconstruction of PepMV strain/host over the maximum clade credibility tree of 1,000 trees (rescaled to reflect median node heights for the contained clades) extracted from the BEAST analysis described below and comparing this number of steps to that for the same character reshuffled 1,000 times, while keeping the proportion of states constant, utilizing Mesquite v.2.75 (<http://mesquiteproject.org/mesquite/>). The null hypothesis of phylogenetic random distribution was rejected if the observed state distribution was outside the 95% confidence interval (CI) of the randomized state distribution, which indicated the presence of a phylogenetic signal.

The ancestral states of the host species and PepMV strain were then inferred using the Bayesian Markov chain Monte Carlo (MCMC) method available in BEAST v.1.7.5 (37). The utilized data set was run using the $GTR+I+\Gamma_4$ model. A relaxed (uncorrelated, log-normal) molecular clock, which allows rate variation among branches in the phylogeny, was assumed. Finally, a flexible Bayesian skyline model as a coalescent prior was used, as estimating demographic parameters was not the aim of this study. The BEAST analysis was run until all relevant parameters con-

verged, with 10% of the MCMC chains discarded as burn-in. TreeAnnotator v.1.7.5 (37) was used to construct a maximum clade credibility tree, which was assumed in estimating posterior distributions for ancestral states. Tree topology and ancestral state posterior probabilities were drawn from 2,000 trees generated in the BEAST analysis. Ancestral state reconstructions using ML methods as implemented in Mesquite yielded similar results. For simplicity, only Bayesian reconstructions are presented.

Construction of biologically active cDNA clones of PepMV isolates. Biologically active clones of PepMV isolates Chi2.9 and Tor9 were obtained essentially as described by Hasiów-Jaroszewska et al. (38) The complete genome was amplified by two-step RT-PCR using the SuperScript III high-yield reverse transcriptase and a SuperMix high-fidelity DNA polymerase with primers UTR5F and UTR3R. Primer UTR5F had in its 5' end the sequence of the T7 promoter, and primer UTR3R had in its 3' end a NotI restriction site. The resulting amplicon was cloned in the pCR-XL-Topo vector (Life Technologies). Thus, plasmids pChi2.9 and pTor9 were obtained. Infectious RNA was obtained from these plasmids after linearization with NotI and transcription with T7 RNA polymerase in the presence of a Cap analogue. RNA was inoculated into *Nicotiana benthamiana* Domin plants, infection was checked by DAS-ELISA, and virus particles were purified as described above for bioassays.

Biological characterization of PepMV isolates. The host ranges of isolates Chi2.9 and Tor9 derived from infectious clones were explored by inoculating 10 μ l of a 20-mg/ml suspension of virus particles in phosphate buffer into the cotyledons or first leaves of a panel of 14 species from the *Solanaceae*. At least four plants per species were inoculated. At 15 and 30 dpi, infection in the inoculated or systemically infected leaves, respectively, was analyzed by DAS-ELISA. Systemic symptoms at 60 dpi were rated.

Within-host multiplication was estimated in some host plant species as virus accumulation. Virus accumulation was quantified in each plant at 30 dpi in pools of all systemically infected leaves by quantitative real-time RT-PCR of total nucleic acid extracts that were obtained using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. For each sample, 58 to 129 ng of total RNA was utilized with Brilliant III Ultra-Fast SYBR green QRT-PCR Master Mix according to the manufacturer's recommendations (Agilent Technologies, CA, USA) in a final volume of 10 μ l. Assays were performed in triplicate on a LightCycler 480 II real-time PCR system (Roche, IN, USA). Relative levels of viral RNA were deduced from standard curves produced using a set of serial dilutions of plant material and purified viral RNAs of isolate Tor9 (PES) and reference isolates Mu07-20 (EU) and Al08-66 (CH2), obtained by us from single-infected tomato plants in southeastern Spain and characterized on the basis of nucleotide sequence determination of various genomic regions (not shown). In order to quantify virus accumulation in the various samples, the following primers were designed: qPCRPEF (TCAGTGGCTACCCCAACTGAA) and qPCRPEER (CGATCAAATTGTGCAGCTAGG), amplifying 202 nucleotides (nt) of the CP gene of PES isolates (GenBank accession no. [HG000317](https://www.ncbi.nlm.nih.gov/nuccore/HG000317)); qPCRCH2F (TGCTGAAATTGAGGCCCTTGG) and qPCRCH2R (AGTGCACGTCTAGACAAAGCA), amplifying 170 nt of the CP gene of CH2 isolates (GenBank accession no. [DQ000985](https://www.ncbi.nlm.nih.gov/nuccore/DQ000985)); and qPCREUF (GCAAATCTTCACCGCTATGG) and qPCREUR (TGATTCGGTCAAACCTGAGCAG), amplifying 173 nt of the CP gene of EU isolates (GenBank accession no. [AJ438767](https://www.ncbi.nlm.nih.gov/nuccore/AJ438767)). Reactions with mixtures including no template and no reverse transcriptase were included in each trial. Thermal parameters for RT-PCR amplification were 50°C for 10 min, 95°C for 3 min, and 40 cycles of 95°C for 5 s and 60°C for 10 s. Dissociation curves were generated to ascertain that only a single product was produced in each case.

Statistical analyses. Incidences of PepMV according to population, location, or host plant species were compared by association tests (χ^2). Analysis of aggregation of infected plants in each population was performed by the run test for dichotomized data (39). Generalized linear models (GLM) assuming a binary logistic distribution were used to test

differences in the efficiency of mechanical inoculation between PepMV isolates. GLM were used to analyze the differences in virus accumulation in the different host plant species of the assayed virus isolates. The presence of outliers, which potentially introduce a bias in GLM analyses, was detected by calculating the studentized residual for each data point, dividing the residual by its standard deviation. Values outside the 95% confidence interval of the Student *t* distribution drawn with all of the studentized residuals were considered outliers (39). Host species and virus isolate were considered fixed factors in the GLM analyses. To determine whether values of analyzed traits were significantly different among classes within each factor, least significant difference (LSD) analyses were employed in all cases (39). Statistical analyses were performed using the statistical software package SPSS 17.0 (SPSS Inc., IL, USA).

Nucleotide sequence accession numbers. The CP gene nucleotide sequences obtained in this work are available from the EMBL data bank under accession numbers [HG000306](#) to [HG000320](#). Full-length nucleotide sequences of the three sequenced isolates are available from the EMBL data bank under accession numbers [HG313805](#) to [HG313807](#).

RESULTS

Incidence of PepMV infection in natural populations of wild tomatoes in Peru.

In March 2008 we conducted a survey for PepMV infection in natural populations of wild tomato relatives (*Solanum* spp.) in the provinces of Arequipa and Moquegua in southern Peru. Twelve populations at nine different locations, spanning the distribution of different *Solanum* species and different ecological environments, were visited (Table 1). Populations varied considerably in size, from about 1,000 plants (Chiguata 1) to fewer than 20 plants (e.g., Mollendo 1 and 2, Cerro Verde 1, and Torata), and showed a highly aggregated spatial distribution. A total of 104 plants were sampled, belonging to four species: *S. peruvianum* (74 plants), *S. chilense* (14 plants), *S. pimpinellifolium* (14 plants), and *S. pinatifidum* (2 plants). The PepMV incidence was about 27% (28/104 infected plants) (Table 1). The incidence did not vary significantly according to population ($\chi^2_{11} = 7.44$, $P = 0.767$), host plant species, or location ($\chi^2 < 0.33$, $P > 0.882$) (Table 1). The small size of the populations did not allow the analysis of the spatial distribution of infected plants except for the Chiguata 1 and Chiguata 2 populations, in which this distribution showed aggregation ($[E(U)] > U$, with $P < 0.05$ in ordinary run analyses).

Molecular characterization and genetic variation of PepMV isolates from wild tomatoes in southern Peru.

The CP gene was successfully RT-PCR amplified for 15 field-infected plants, and its nucleotide sequence was determined (Table 2). Sequences were highly similar for all 15 isolates, with only three polymorphic sites at positions 468, 495, and 712 of the CP gene and two other polymorphic sites at positions 725 and 726 (in the 3' UTR for most isolates). All mutations were silent except the mutation U→A at position 712, which resulted in the change Stop→K and in the addition of four amino acids to the 268 of the PepMV CP. Population diversity measured as nucleotide diversity was very low, about 0.001. Nucleotide polymorphisms defined five haplotypes. Haplotype H1 represented 73% of the population (11/15 analyzed plants) and was found in all locations and in different host species. Haplotypes H2 to H5 occurred only once each in the sample and were found in different populations (Table 2).

Three out of the 15 PepMV isolates with sequenced CPs were transferred to tomato plants for multiplication and molecular characterization. These isolates were selected because they were collected from different host plant species and different regions in

TABLE 2 Nucleotide polymorphisms at the coat protein gene and distribution of haplotypes in the PepMV population

Isolate	Origin		Nucleotide at position:			Haplotype
	Population	Host species	468	495	712	
Chi1.8	Chiguata 1	<i>S. peruvianum</i>	U	U	U	H1
Chi2.5	Chiguata 2	<i>S. peruvianum</i>	U	U	U	H1
Cvd2.6	Cerro Verde 2	<i>S. pimpinellifolium</i>	U	U	U	H1
Mol1.3	Mollendo 1	<i>S. chilense</i>	U	U	U	H1
Mol2.2	Mollendo 2	<i>S. chilense</i>	U	U	U	H1
Yur1.2	Yura 1	<i>S. peruvianum</i>	U	U	U	H1
Yur1.5	Yura 1	<i>S. peruvianum</i>	U	U	U	H1
Sam2.5	Samegua 2	<i>S. peruvianum</i>	U	U	U	H1
Sam2.6	Samegua 2	<i>S. peruvianum</i>	U	U	U	H1
Ceb3	Cerro El Baúl	<i>S. peruvianum</i>	U	U	U	H1
Tor9	Torata	<i>S. peruvianum</i>	U	U	U	H1
Ceb6	Cerro el Baul	<i>S. peruvianum</i>	U	C	U	H2
Chi2.9	Chiguata 2	<i>S. pimpinellifolium</i>	C	U	U	H3
Mol2.8	Mollendo 2	<i>S. chilense</i>	U	U	A	H4
Tor13	Torata	<i>S. peruvianum</i>	C	U	A	H5

southern Peru and represented different CP haplotypes. Isolates Yur1.5 and Tor9 (both H1) were originally from *S. peruvianum* plants collected from the Yura 1 and Torata populations, respectively, and isolate Chi2.9 (H3) was originally from an *S. pimpinellifolium* plant collected from the Chiguata 2 population. The nucleotide sequences of their full genomes were determined and showed a high pairwise sequence similarity of above 99%. Similarity was much lower with representative isolates of the four recognized strains: about 86% with US1 and between 78% and 82% with EU, LP, and CH2. When the different genomic regions were compared separately, similarity between isolates Chi2.9, Yur1.5, and Tor9, and the previously recognized strains was highest at the 3' UTR with US1 (94.2%) and at the TGB2 and TGB3 open reading frames (ORFs) with the US1 and EU strains (86 to 92%). Values for the 5' UTR and for the three other ORFs were similar to those for the whole genome (not shown). An analysis to identify possible recombination events between isolates Chi2.9, Yur1.5, and Tor9 and reported full-length sequenced isolates of the four recognized PepMV strains failed to detect any recombination breakpoint (not shown). Thus, isolates Chi2.9, Yur1.5, and Tor9 belong to a new strain of PepMV that we named the PES strain, after the southern Peru region of isolation. Phylogenetic analyses showed that the new PES strain clustered significantly with US1 and that strains PES and US1 grouped together with CH2 isolates. The EU and LP sequences formed a separate cluster (Fig. 1). A phylogenetic tree obtained using the 15 CP sequences plus representative isolates of all other PepMV strains mimicked the topology obtained using complete nucleotide genomic sequences (not shown). Hence, the sampled PepMV population in wild tomato relatives in southern Peru consisted only of isolates of the PES strain and was genetically a single, undifferentiated, highly homogeneous population.

Biological characterization of isolates of PepMV strain PES.

As there is no known local lesion host for the biological cloning of PepMV isolates (6) and the sampled field-infected plants could be mixed infected with other viruses, for the biological characterization of the new PES strain of PepMV, full-length, biologically active cDNA clones were obtained for the above-described isolates

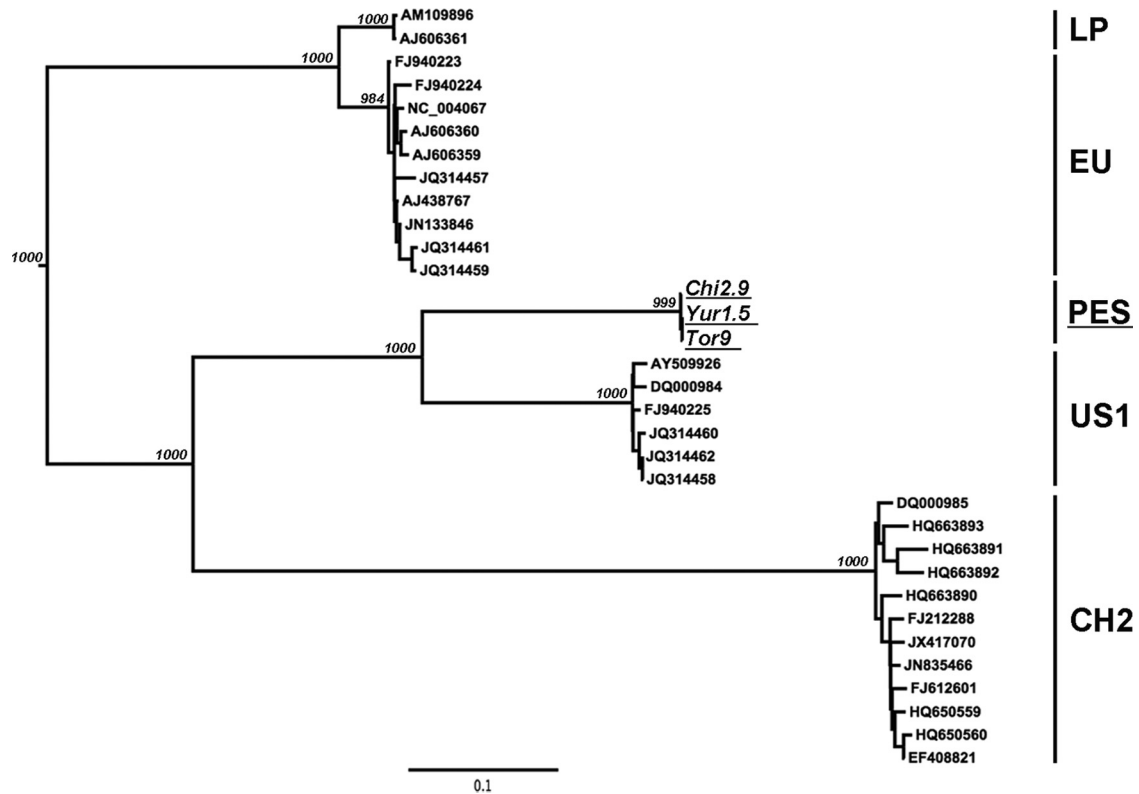


FIG 1 Maximum-likelihood phylogenetic tree of *Pepino mosaic virus* isolates, showing five recognized strains. Significance of nodes in a bootstrap analysis with 1,000 replicates is indicated. All isolates for which the complete sequence of the genome has been reported were included in the analysis. The tree was midpoint rooted.

Chi2.9 and Tor9. Host range tests using a panel of 14 species from the *Solanaceae* showed that PES PepMV isolates had a host range very similar to that of isolates Mu07-20 and Al08-66, representative of the EU and CH2 strains, respectively (Table 3), with the efficiency of mechanical inoculation in each host species being not significantly different between PepMV isolates ($\chi^2 < 1.77$, $P >$

0.184). All assayed isolates infected systemically and with high efficiency *Datura stramonium* L., *S. lycopersicum*, *S. muricatum*, *S. pimpinellifolium*, *Solanum melongena* L., *Nicotiana clevelandii* A. Gray, and *N. occidentalis*. Systemic infection also occurred in the wild tomato species *S. peruvianum*, *S. chilense*, and *S. habrochaites*, except that isolate Al08-66 (CH2) did not infect the last species.

TABLE 3 Host range and symptomatology of PepMV isolates

Host species	% of plants infected or symptomatic with isolate (strain) ^a :							
	Chi2.9 (PES)		Tor9 (PES)		Mu07-20 (EU)		Al08-66 (CH2)	
	Infection	Symptoms	Infection	Symptoms	Infection	Symptoms	Infection	Symptoms
<i>Solanum lycopersicum</i>	100	100 C	100	100 C	100	25 AS, 75 LC	100	25 AS, 75 C/LC
<i>Solanum peruvianum</i>	50	50 AS	50	50 AS	100	100 AS	25	25 AS
<i>Solanum pimpinellifolium</i>	100	100 C/N	100	100 C/N	100	100 C	100	100 C
<i>Solanum chilense</i>	0		50	50 AS	0		25	25 AS
<i>Solanum habrochaites</i>	25	25 AS	50	50 AS	25	25 AS	0	
<i>Solanum muricatum</i>	100	50 C/W, 50 C	75	25 LC/W, 50 C	75	50 C, 25 N	100	50 C, 50 C/LC
<i>Solanum melongena</i>	100	100 N	100	50 AS, 50 W	100	75 AS, 25 C	100	100 AS
<i>Nicotiana benthamiana</i>	25	25 C/LC	0		75	75 C/LC	50	50 AS, 50 LC
<i>Nicotiana clevelandii</i>	100	25 C, 75 LC	100	100 C/LC	100	100 C/LC	75	75 C/LC
<i>Nicotiana occidentalis</i>	100	100 LC/N	100	100 C/LC	100	100 LC	100	100 C/N
<i>Nicotiana tabacum</i>	50	50 AS	0		25	25 AS	0	
<i>Capsicum annuum</i>	0		0		0		0	
<i>Datura stramonium</i>	100	25 C/LC, 25 C/W, 25 C, 25 C/LC/W	100	100 C/LC/W	100	100 C/LC	100	100 C/N
<i>Physalis floridiana</i>	0		0		0		0	

^a Numbers are the percentage of infected or symptomatic plants over four plants inoculated. Symptoms: AS, asymptomatic; C, chlorosis; LC, leaf curl; N, necrosis; W, wilting.

TABLE 4 Accumulation of PepMV isolates in different *Solanum* species

Solanum species and cultivar	μg viral RNA/g total RNA for isolate ^a :			
	Mu07-20 (EU)	Al08-66 (CH2)	Chi2.9	Tor9
<i>S. lycopersicum</i> Marglobe	0.269 ± 0.076	0.049 ± 0.011	0.509 ± 0.046	0.316 ± 0.058
<i>S. lycopersicum</i> MoneyMaker	0.415 ± 0.056	0.168 ± 0.052	0.615 ± 0.117	0.613 ± 0.127
<i>S. pimpinellifolium</i>	0.284 ± 0.064	0.103 ± 0.029	1.592 ± 0.276	1.121 ± 0.149
<i>S. chilense</i>	0.117 ± 0.026	0.016 ± 0.006	1.402 ± 0.272	0.337 ± 0.124
<i>S. peruvianum</i>	0.455 ± 0.075	0.018 ± 0.005	0.954 ± 0.149	0.849 ± 0.146

^a Data are means ± standard errors for at least 5 plants.

Efficiency of mechanical inoculation was lower in the wild *Solanum* species than in the previous group of hosts ($\chi^2 = 6.45$, $P = 0.011$). No isolate systemically infected *Capsicum annuum* or *Physalis floridiana* Rydb. Infection of the systemic hosts was mostly asymptomatic or resulted in mild symptoms of chlorosis, leaf distortion, or leaf curl. Symptoms were generally more severe in cultivated than in wild *Solanum* host species (Table 3).

Differential accumulation of PepMV isolates in wild and domestic tomatoes. Virus accumulation of PepMV isolates Chi2.9, Tor9, Mu07-20, and Al08-66 in three wild tomato species (*S. peruvianum*, *S. chilense*, and *S. pimpinellifolium*) and in two cultivars of domestic tomato (*S. lycopersicum* cv. Marglobe and *S. lycopersicum* cv. MoneyMaker) was determined via real-time RT-PCR (Table 4). GLM analyses indicated that virus accumulation significantly depended on the host species ($F_{4,158} = 6.56$, $P < 1 \times 10^{-4}$), on the virus isolate ($F_{3,158} = 38.38$, $P < 1 \times 10^{-4}$), and on the species-per-isolate interaction ($F_{11,158} = 4.25$, $P < 1 \times 10^{-4}$). Since we chose *Solanum* species to represent wild and domesticated hosts, we also performed GLM analyses with nesting of host species to host status (i.e., wild or domesticated). Again, virus accumulation varied according to virus isolate ($F_{3,158} = 9.35$, $P = 2 \times 10^{-3}$) and to the interaction virus isolate per host species ($F_{11,158} = 4.25$, $P < 1 \times 10^{-4}$), but it did not vary according to host species ($F_{4,158} = 1.11$, $P = 0.384$).

As we observed an interaction between virus strain and *Solanum* species on PepMV multiplication, even when correcting for the effect of plant domestication status, we analyzed next if there was evidence of PepMV adaptation in the wild and/or the domestic hosts. For this analysis, two classes of virus isolates were considered: (i) isolates from wild tomatoes (isolates Chi2.9 and Tor9) and (ii) isolates from tomato crops (isolates Mu07-20 and Al08-66). Also, two classes of hosts were considered: (i) wild tomato species (i.e., *S. peruvianum*, *S. chilense*, and *S. pimpinellifolium*) and (ii) the cultivars of *S. lycopersicum* (Marglobe and MoneyMaker) as representatives of the major crop (tomato) in which PepMV has recently emerged as an important pathogen. Analyses of the data presented in Fig. 2 showed that accumulation of PepMV isolates from wild hosts was higher in wild than in domestic tomatoes ($F_{1,83} = 14.28$, $P < 1 \times 10^{-4}$), while tomato isolates showed a nonsignificant trend toward higher accumulation in cultivated than in wild *Solanum* species ($F_{1,75} = 2.59$, $P = 0.112$). Also, accumulation of PepMV isolates from domestic tomato was lower than accumulation of PepMV isolates from wild tomatoes in both wild ($F_{1,99} = 65.17$, $P < 1 \times 10^{-4}$) and domestic ($F_{1,59} = 18.63$, $P < 1 \times 10^{-4}$) tomatoes. These analyses show strong evidence of host adaptation for PepMV isolates from wild hosts, and they suggest adaptation to tomato of tomato isolates. Also, they

suggest that there is a trade-off between virus fitness in wild tomato species and in domestic tomato.

Host ancestral state reconstruction of PepMV isolates. Our results on PepMV incidence and virus multiplication as well as previously reported epidemiological data are compatible with PepMV emergence in tomato being associated with spread from wild *Solanum* species. To further analyze this possibility, we performed an ancestral state reconstruction considering as state the PepMV strain, since LP and PES strains are largely associated with wild hosts and EU and CH2 with domestic tomatoes. To do so, complete genomic sequences of PepMV PES, CH2, and EU isolates (Fig. 3) (i.e., of those strains that we had compared for host range and within-host multiplication) and LP isolates that contained the oldest known PepMV sequence were used.

As the PepMV ancestral state reconstruction considered the time scale of virus evolution, we first assessed the strength of temporal and state-related phylogenetic signals in the utilized data set, which is necessary for a meaningful analysis. A significant root-to-tip correlation of sampling time versus genetic distance ($r = 0.60$; $P = 1 \times 10^{-4}$) was observed. Analyses performed using only those sequences collected between 1999 and 2010 yielded similar results ($r = 0.58$; $P = 1 \times 10^{-4}$). Therefore, the utilized PepMV data set contained sufficient temporal structure for reliable estimation of the PepMV evolutionary time scale. This was confirmed by the significantly smaller mean and larger HPDs of the substitution rate estimates obtained for randomized data sets compared with those from the real data (not shown). In addition, the null hypothesis of phylogenetic random distribution of PepMV strain state was rejected by parsimony reconstruction of trait evolution because the observed distribution (4 steps) was outside the 95% CI of the randomized state distribution (mean = 12.8, median = 17, $CI_{Lower} = 8.2$, $CI_{Upper} = 21.3$), indicating the presence of a PepMV state-related phylogenetic signal.

Phylogenetic trees annotated with the node state (i.e., the PepMV strain with the highest posterior probability [PP] at each node) indicated that the most probable ancestor of all PepMV sequences belonged to the LP strain (PP = 0.55), and was thus from a wild host, with the PP of any other strain being lower than 0.15. Interestingly, the cluster grouping sequences of PES and

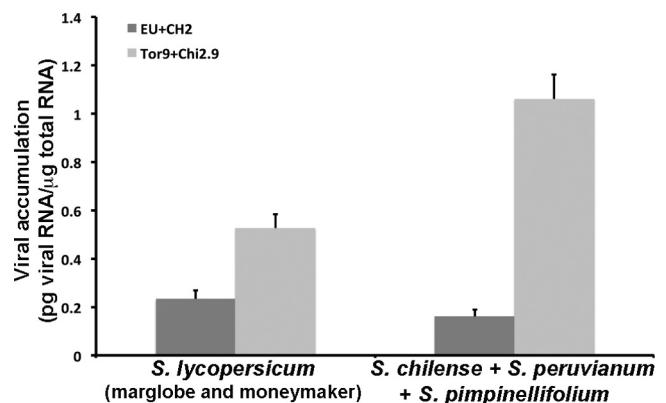


FIG 2 Accumulation of *Pepino mosaic virus* isolates in different host plant species. The accumulation of isolates from tomato crops in Spain (dark gray bars) or from wild *Solanum* spp. in Peru (light gray bars) was compared in two different host types: tomato (*S. lycopersicum* cv. Marglobe and cv. MoneyMaker) and wild tomato (*S. peruvianum*, *S. pimpinellifolium*, and *S. chilense*). Data are means and standard errors from at least 5 infected plants.

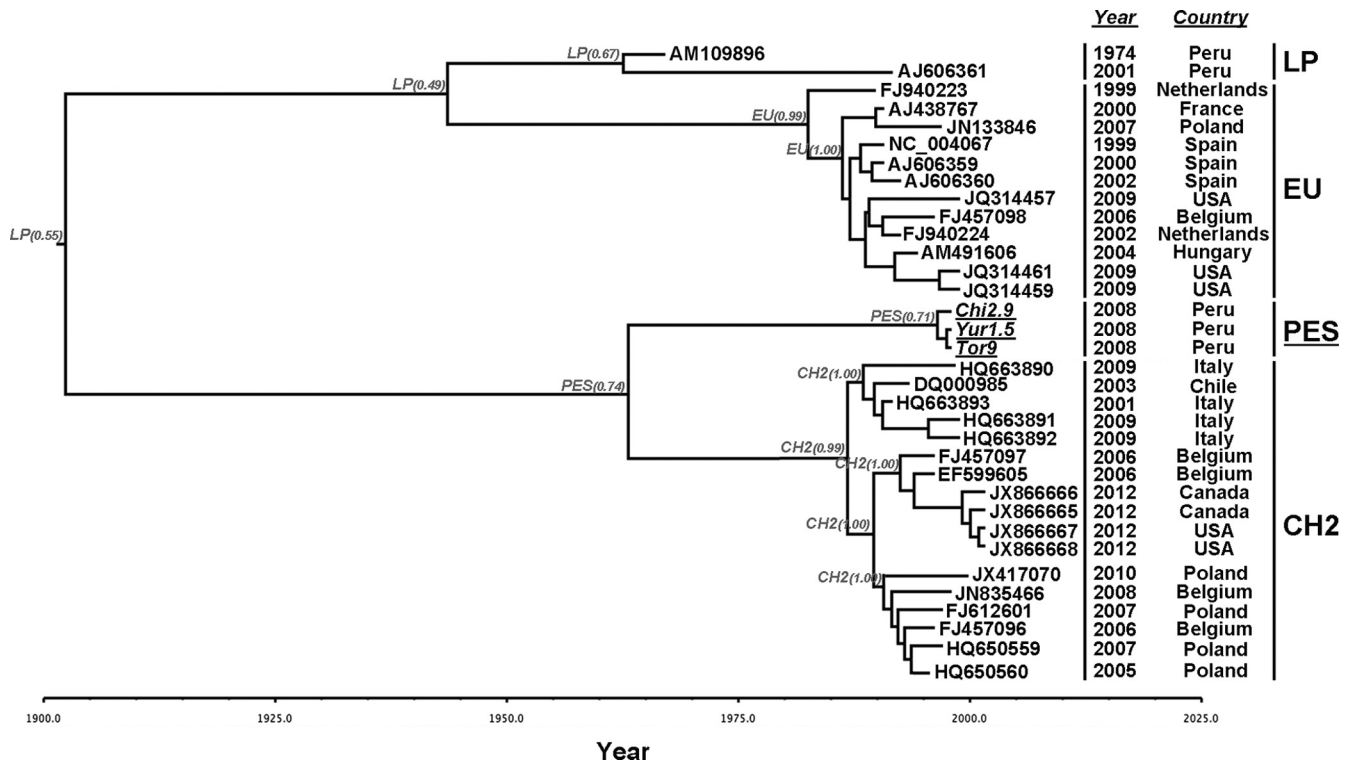


FIG 3 Phylogenetic tree with ancestral state reconstruction of PepMV isolates. Branch tip times reflect the times of viral sampling. The tree is automatically rooted through the use of a relaxed molecular clock, and the total depth of the tree is the time to the most recent common ancestor. Nodes are labeled with the most probable state, and its posterior probability is shown in parenthesis. Tip names indicate the GenBank accession number. The two columns on the right indicate the year of isolation and country of origin of the PepMV isolate that originated each sequence. All sequences were obtained from PepMV isolates infecting *S. lycopersicum*, with the exceptions of sequences AJ606361, Yur1.5, and Tor9 (obtained from *S. peruvianum*), sequence Chi2.9 (obtained from *S. pimpinellifolium*), and sequence AM109896 (obtained from *S. muricatum*).

CH2 isolates had a common ancestor from the PES strain, again associated with wild hosts (PP = 0.74) (Fig. 3), with the PP of any other strain being lower than 0.07. As expected, the ancestral state reconstruction using host species as state yielded comparable results. Importantly, ancestral state reconstruction based on ML methods yielded similar results. In addition, the Bayesian node-annotated trees showed topologies similar to those of trees obtained by ML, either midpoint rooted or rooted using the *Narcissus mosaic virus* type sequence (trees are available upon request). Thus, our phylogenetic reconstructions appear to be robust regardless of the methodology or the rooting method utilized.

DISCUSSION

Emergent viral diseases of plants often have severe negative impacts in agriculture and forestry and may result in dramatic changes in the species composition and dynamics of wild plant communities. Human activities resulting in the alteration of natural ecosystems, agriculture intensification and spread, or increased connectivity among plant populations due to trade, as well as global climatic change, are considered to presently increase the risk of virus emergence (2, 3, 5, 40–43). It is thus necessary to understand how multiple ecological, evolutionary and epidemiological factors contribute to plant viral disease emergence (2–4). With this aim, we have attempted to analyze the causes leading to the emergence of a virus that became a worldwide threat to tomato production in the recent past, PepMV (6, 7). Most work aimed at understanding plant virus emergence has focused on either the a

posteriori identification of ecological and epidemiological causes or the experimental evolutionary analysis of host adaptation (3, 4). We followed a different approach: we evaluated the ecological and evolutionary risk of emergence of a PepMV strain as yet unreported in crops, we compared the relevant traits of this strain and of strains infecting tomato crops, and we attempted to trace the origin of the PepMV epidemic by comparative genomics analyses.

Spread from wild hosts is often the first step in virus emergence (43–45). This could also have been the case in PepMV emergence, as (i) the virus was detected infecting wild tomato species in central and southern Peru (28), (ii) Peru and Chile are important regions of seed production for seed companies (46), and (iii) some strains of PepMV were first identified in tomato seed produced in this region (17). Thus, we surveyed for PepMV infection in natural populations of several wild tomato species in different habitats in southern Peru, finding a high prevalence of infection by a new strain of PepMV that we named PES. Note that the prevalence reported here is likely a lower threshold, as PepMV detection was probably inefficient due to suboptimal survival of samples. The high prevalence of infection at all locations, including in quite small plant populations, and the aggregated distribution of infected plants observed in large host populations are compatible with reported seed and contact transmission of PepMV (6, 7, 13). A high incidence of infection and a large host range within the *Solanaceae* family of PepMV strain PES isolates would favor virus

spread to local solanaceous crops, such as pepino or tomato, either directly or through weeds such as local species of *Datura* or *Nicotiana* (47, 48). It should be pointed out that wild tomato species also occur frequently as weeds in irrigated crops in southern Peru (49), a factor further favoring virus spread to crops. Importantly, our phylogenetic reconstruction of the PepMV ancestral state indicated that the deepest node in the phylogeny and those defining the LP-EU and the PES-CH2 clades were associated with viruses infecting wild hosts. Therefore, the analyses performed with the current sampled diversity of PepMV support the hypothesis of an origin of the PepMV epidemic as a consequence of virus spread from a wild host. Obviously, we cannot discard that there is still undiscovered PepMV genetic diversity, which is a clear limitation of our phylogenetic analyses. Future surveys will allow a finer reconstruction of PepMV emergence.

After spread from wild hosts has resulted in the infection of a few individuals of the new host, viruses must adapt to establish a productive infection for successful transmission in the new host population. Host adaptation is facilitated if the original virus population has enough genetic variation to contain variants with a positive fitness in the new host, without the need of generating these variants anew through mutation (4, 50). The PepMV PES population infecting wild tomatoes would not fulfill this condition, as it showed very low genetic variation, a trait shared with single-strain populations of PepMV infecting tomato crops elsewhere (10, 20, 21). However, low genetic diversity should not be a hindrance for adaptation to tomato or pepino crops: PepMV PES isolates appear to be generalists, being able to successfully infect different wild and domestic species of at least two genera of the subfamily *Solanoidea*, i.e., *Solanum* and *Datura* (Table 3). Hence, emergence in pepino and/or tomato would not involve a host range expansion, as is often the case for emerging plant pathogens (2, 3). Finally, the characterized PepMV PES isolates multiplied to high levels in both wild and domestic tomatoes, which could favor their future emergence in tomato crops. Thus, all our observations point to a high risk of an emergence of PepMV PES as a crop pathogen.

The more efficient multiplication in wild than in cultivated hosts of PepMV PES isolates supports a scenario of adaptation to their wild hosts (Fig. 2). Conversely, tomato isolates of PepMV strains EU and CH2 show a trend toward more efficient multiplication in tomato than in wild *Solanum* species, which is also suggestive of a process of host adaptation. Note also that the phylogeny of PepMV, in which isolates clustered mostly according to their host species (Fig. 1 and 3), may also be indicative of host adaptation. We cannot discard other scenarios. For instance, the ancestor of the EU or CH2 strains may have had high fitness on wild tomato hosts and with exaptations to cultivated tomato. Also, increased fitness on cultivated tomato may have occurred without affecting the low fitness of EU or CH2 on wild hosts. However, our data suggest that adaptation is the most likely mechanism involved in PepMV emergence.

Host adaptation in viruses is often conditioned by across-host trade-offs in fitness that result in evolution toward specialism (4, 51). In our analyses, a fitness trade-off is apparent both for tomato and wild host viral strains between *S. lycopersicum* and the wild tomato species of *Solanum*. Evidence of across-host fitness trade-offs for plant viruses is abundant from experimental evolution of serially passaged viruses in different hosts (see, e.g., references 52 to 55) but rarely derives from the analysis of field isolates (but see

references 56 to 58). It should be underscored that the support for across-hosts fitness trade-offs reported here for PepMV isolates derives from the analysis of field isolates that were subject to only one passage in the same multiplication host and is highly coherent with experimental results with other systems. Although, given the observed trade-off, a single passage through domestic tomato may lead to fixation of mutations increasing virus fitness in this host, we followed a procedure which minimizes this effect (see Materials and Methods). More importantly, comparison of CP sequences of 10 PepMV PES isolates before and after passage showed no nucleotide changes (not shown).

In summary, the results from this work contribute to understanding the pathway to emergence of PepMV as an important pathogen of tomato crops worldwide. From an initial situation in which different PepMV strains would be found infecting wild *Solanum* spp. in Peru, virus spread would have occurred to tomato directly (e.g., for the CH2 strain) or perhaps through previous emergence in *S. muricatum* (for the EU strain). The ability of wild *Solanum* isolates to efficiently multiply in different *Solanum* species, shown for the LP and PES strains (19, 28; this work) would favor the establishment of PepMV in the new crop hosts as an epidemic pathogen. Last, adaptation to tomato, possibly traded off against adaptation to wild *Solanum* species, would feed back tomato epidemics and isolate tomato populations from those in other hosts.

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