Multihost Experimental Evolution of a Plant RNA Virus Reveals Local Adaptation and Host-Specific Mutations

Stéphanie Bedhomme,*1 Guillaume Lafforgue,1 and Santiago F. Elena1,2

1Consejo Superior de Investigaciones Científicas-UPV, Instituto de Biología Molecular y Celular de Plantas, València, Spain
2The Santa Fe Institute, Santa Fe, New Mexico
*Corresponding author: E-mail: stebed@upvnet.upv.es.

Abstract

For multihost pathogens, adaptation to multiple hosts has important implications for both applied and basic research. At the applied level, it is one of the main factors determining the probability and the severity of emerging disease outbreaks. At the basic level, it is thought to be a key mechanism for the maintenance of genetic diversity both in host and pathogen species. Using Tobacco etch potyvirus (TEV) and four natural hosts, we have designed an evolution experiment whose strength and novelty are the use of complex multicellular host organism as hosts and a high level of replication of different evolutionary histories and lineages. A pattern of local adaptation, characterized by a higher infectivity and virulence on host(s) encountered during the experimental evolution was found. Local adaptation only had a cost in terms of performance on other hosts in some cases. We could not verify the existence of a cost for generalists, as expected to arise from antagonistic pleiotropy and other genetic mechanisms generating a fitness trade-off between hosts. This observation confirms that this classical theoretical prediction lacks empirical support. We discuss the reasons for this discrepancy between theory and experiment in the light of our results. The analysis of full genome consensus sequences of the evolved lineages established that all mutations shared between lineages were host specific. A low degree of parallel evolution was observed, possibly reflecting the various adaptive pathways available for TEV in each host. Altogether, these results reveal a strong adaptive potential of TEV to new hosts without severe evolutionary constraints.

Key words: experimental evolution, local adaptation, Tobacco etch virus, parallel evolution, full genome sequence, virulence evolution.

Introduction

The environment of a pathogen and consequently, the source of selection pressures is, for the majority of its life cycle, its host. The particular host environment presents different forms of heterogeneity, even on short evolutionary time scales: from different cell types within a tissue to different species (Thomas et al. 2002). Multihost pathogens, which encounter the latter, more extreme case of heterogeneity, should be able to cope with different biotic conditions but also, due to their parasitic reproduction strategy, exploit different cellular mechanisms and resources for their own growth and reproduction. The adaptation to various hosts is supposed to be constrained by the fact that mutations advantageous in one environment tend to be disadvantageous in another environment (Gandon 2004).

Adaptation to multiple hosts has important implications both for applied and basic research. In recent years, emerging diseases have represented an important threat both for applied and basic research. In recent years, emerging diseases have represented an important threat. Taking into account the potential for local adaptation, for any multihost pathogen specific lineages are likely to fall along a gradient, which in principle ranges from absolute specialists, those pathogens only able to infect and reproduce in
a single host, to absolute generalists, able to infect and reproduce equally well within any species of the host range. The position of a particular pathogen lineage along this gradient depends on: 1) the environmental factors, 2) the genetic diversity within each lineage, and 3) the global fitness of each evolutionary strategy. First, the environmental factors affecting the evolutionary fate of a multihost pathogen are the frequency and distribution of each species of the host range, which strongly influence the migration rate between host species (Woolhouse et al. 2001; Ravigne et al. 2009). For example, for a plant pathogen, a monoculture field is clearly a situation favoring specialist genotypes, whereas a diverse ecosystem is expected to favor generalist genotypes. An additional environmental factor for vector-transmitted pathogens is the availability and the specialization of the vectors. Indeed, vectors usually feed on hosts and themselves have a preference toward one or more hosts, thus strongly influencing the probability of transmitting the parasite from one species to another (Woolhouse et al. 2001). Second, the genetic diversity of a pathogen population within one host species conditions the chances of appearance and selection of mutations that allow for a sufficient infectivity and reproduction rate to maintain the pathogen within another host species. The genetic diversity itself depends on population genetic parameters such as the mutation rate, the reproduction rate, the intrahost effective population size, and the population structure. Third, generalist evolutionary strategies have been predicted to be disadvantaged for three distinct reasons: 1) antagonistic pleiotropy, that is, the fact that mutational effects are negatively correlated between hosts, is thought to play a preponderant role in many cases (Via 1990; Fry 1996; Gandon 2004). 2) Since generalists alternate between hosts, some pathogen genes can be expressed during infection in one host and not expressed in another host. The absence of expression part of the time implies the absence of selection acting on these loci and the potential for accumulation of mutations, which will be deleterious when expressed in the other host (Kawecki 1994). 3) The alternation between hosts by generalists implies that selection pressures specific to a particular host act only part of the time, slowing down the rate of allele fixation and elimination compared with that of a specialist (Whitlock 1996). Field studies have shown the disadvantage of generalists in a diversity of systems (e.g., Poulin 1998, fish parasites; Poulin and Mouillot 2004, helminth parasites of birds; Malpica et al. 2006, viruses and weeds; Straub et al. 2011, endoparasitoids and aphids). In other systems, however, the generalist strategy seems to be favored (e.g., Krasnov et al. 2004, ectoparasitic fleas and small mammals; Hellgren et al. 2009, *Plasmodium* sp. and birds). This discrepancy between theoretical predictions and some field data is likely to be due to the influence of other factors (environmental and genetic diversity) and to the lack of universality of the costs of adaptation (Hereford 2009).

An efficient way to investigate the factors and mechanisms of specialization and the implications for the evolution of specialist and generalist strategies is to use experimental evolution (Kawecki and Ebert 2004). It allows explicitly addressing hypotheses about the role of particular ecological and genetic factors that promote or hinder local adaptation, maintaining other factors fixed. A number of experimental evolution studies on specialization and generalist/specialist strategies have already been published using a variety of experimental systems (e.g., Turner and Elena 2000; *Vesicular stomatitis virus* (VSV)/hamster, human, or canine cell; Magalhaes et al. 2009, spider mites/host plants; Legros and Koella 2010, microsporidia/mosquito). Almost all experimental evolution studies show a pattern of host specialization (Novella et al. 1999; Turner and Elena 2000; Cooper and Scott 2001; Greene et al. 2005; Wallis et al. 2007; Agudelo-Romero et al. 2008; Coffey et al. 2008; Magalhaes et al. 2009; Vasilakis et al. 2009; Legros and Koella 2010; Coffey and Vignuzzi 2011; Deardorff et al. 2011), but only in some cases is specialization accompanied by a cost (Weaver et al. 1999; Turner and Elena 2000; Wallis et al. 2007; Agudelo-Romero et al. 2008; Vasilakis et al. 2009; Legros and Koella 2010; Deardorff et al. 2011). Finally, evidence for the cost of being a generalist are even less frequent (Coffey et al. 2008; Legros and Koella 2010).

Among the existing experimental evolution studies, those using RNA viruses are overrepresented. There are various reasons for this overrepresentation. First, RNA viruses are likely to evolve in a reasonable experimental time because they have large population sizes, fast replication rates, and a high mutation rate due to the absence of proofreading activity of the RNA polymerase (Elena and Sanjuan 2007). Second, the small genome size allows access to the full genome sequence of the evolved lineages and to reveal the genetic changes that underlie phenotypic evolution. Third, RNA viruses present a large diversity of evolutionary strategies. Examples in animals range from highly specialized viruses, such as the *Poliovirus* (Picornaviridae) that only infects humans, to the *Influenza A virus* (Orthomyxoviridae), able to infect hosts from distant phylogenetic groups (Wolfe et al. 2007). Diversity is also found in RNA plant viruses, with some viruses such as the *Tobacco etch potyvirus* (TEV), which is a relative specialist compared with the *Cucumber mosaic cucumovirus*, known to infect members of 16 different plant families. In this context, a group of RNA viruses that has attracted a lot of attention is the arboviruses (arthropod-borne viruses), whose infective cycle includes obligate alternation between vertebrate and insect hosts. The constraints imposed by the evolutionary trade-offs have been proposed as a likely cause of their relative genetic homogeneity and constancy over time (Woelk and Holmes 2002).

It is important to note that the majority of these experimental evolution studies use cell cultures as “hosts.” Cell cultures are simple homogeneous environments, with no tissue structure and no immune pressure, and probably represent conditions where selection pressures are constant and unidirectional. Actually, it has been shown that local adaptation obtained by evolution in mammalian cell cultures cannot be extrapolated to the corresponding organism (Ciota et al. 2007). Moreover, experimental evolution
conducted in vivo and in the corresponding cell culture lead to very different results (Coffey et al. 2008). Additionally, all the studies cited above compare the outcome of one evolutionary history alternating between two hosts with the two corresponding single-host evolutionary histories. It is thus possible that the results in the alternate evolutionary history are not due to alternation of host environment but are idiosyncratic to this specific treatment. In other words, when technically and experimentally possible, it is desirable to have, on top of the technical “within evolutionary history” replication, different generalist and specialist evolutionary histories. This extra level of replication allows for more robust conclusions on the existence of a specialist–generalist trade-off for a particular pathogen. Among the published evolution experiments with viruses, only four were in vivo experiments (Wallis et al. 2007; Agudelo-Romero et al. 2008; Coffey et al. 2008; Deardorff et al. 2011). Three of these studies were designed to evaluate the evolutionary constraints imposed by vector transmission on adaptation (Wallis et al. 2007; Coffey et al. 2008; Deardorff et al. 2011). The other study used only two hosts and did not contain any evolutionary treatment with host alternation (Agudelo-Romero et al. 2008).

For the present study, we designed an evolution experiment to study local adaptation of TEV to four hosts within its natural host range. The hosts were complete plants, with all the complexity this completeness implies for the dynamics of viral infection. Moreover, we put a special emphasis on the replication of specialist and generalist evolutionary histories: four “single-host” and three “alternate-host” evolutionary histories were experimentally derived with a high “within history” replication level. With this experimental setup, we aimed at collecting data in conditions that were “as natural as possible” and would allow for drawing robust and broad conclusions on generalist and specialist strategies, as well as on the conditions for local adaptation. The infectivity and virulence characteristics of the experimentally evolved lineages were analyzed and their full genome consensus sequences obtained. It has to be noted here that testing for local adaptation, although it is often a useful parameter to understand the implications of the evolutionary and coevolutionary processes in host–pathogen systems.

Materials and Methods

Virus and Plants

Our model system is TEV. TEV is a member of the Potyvirus genus within the Potyviridae family and has a moderately wide host range (Shukla et al. 1994). It has a positive sense single-strand RNA genome of 9.5 kb that encodes a large polyprotein, which is autocatalytically cleaved into ten multifunctional mature viral proteins (Riechmann et al. 1992). Recently, an overlapping open reading frame coding a small additional protein after frameshifting has been discovered (Chung et al. 2008). The genome replication in Potyviridae is performed by a virus-encoded RNA-dependent RNA polymerase that lacks proofreading activity. TEV mutation rate is thus high, estimated to be around $10^{-5}$ to $10^{-6}$ mutations per site and per generation (Tromas and Elena 2010).

Plasmid pMTEV contains the TEV genome (Bedoya and Darós 2010) and was a generous gift by Dr J.-A. Darós. The TEV genome used to generate this clone has been isolated from Nicotiana tabacum (Carrington et al. 1993), and its sequence is published elsewhere (Carrasco, Darós, et al. 2007). A stock of infected tissue was generated before starting the evolution experiment (for details, see supplementary material, Supplementary Material online).

Four host species were used: N. tabacum, Nicotiana benthamiana, Datura stramonium, and Capsicum annuum. They all belong to the Solanacea family, and TEV produces systemic symptoms in all of them. For all the experimental steps, plants were maintained in a greenhouse at 25 °C and a 16 h photoperiod.

Viral Accumulation in Each Host

Before starting the experimental evolution, viral accumulation in each of the four hosts was measured by RT-qPCR (for details, see supplementary material, Supplementary Material online) to ensure that transmission took place when the viral load had reached a plateau and that the quantity of virus used for transmission was equivalent for the four host species. This was done to equalize the size of the transmission bottleneck in every experimental evolution lineage, and thus differences between lineages could not be attributed to differential influence of genetic drift. The obtained accumulation curve indicated that at 7 dpi, viral accumulation had reached a plateau and that viral genomes accumulated at the same level in N. tabacum, N. benthamiana, and D. stramonium (around $2.2 \times 10^7$ viral RNA molecules per 100 ng of total RNA) and at a lower level in C. annuum ($3.2 \times 10^6$ viral RNA molecules per 100 ng of total RNA).

Experimental Evolution

The experimental evolution design contained seven evolutionary histories (fig. 1). In four of them, the viruses were
serially passaged in the same host, hereafter denominated as lineages Nb (\textit{N. benthamiana}), Ds (\textit{D. stramonium}), Ca (\textit{C. annuum}), and Nt (\textit{N. tabacum}). In the three other lineages, the viruses were serially passaged on alternate hosts using the following pairs: (\textit{N. benthamiana}, \textit{N. tabacum}—hereafter NbNt), (\textit{N. tabacum}, \textit{C. annuum}—NtCa), and (\textit{D. stramonium}, \textit{C. annuum}—DsCa). The first type of evolutionary history represents the conditions for the selection of a specialist strategy, whereas the second type is an experimental approximation of conditions selecting for a more generalist strategy. Each evolutionary history was replicated ten times.

To initiate the experimental evolution, a sap was prepared with 300 mg of infected tissue (from the previously described stock) and 450 μl of inoculation buffer. For each replicate, two plants were mechanically inoculated with 5 μl of this sap on one leaf. All lineages were thus started with a genetically homogeneous viral population, and de novo mutation was the only source of raw material on which selection and genetic drift could act (Elena and Lenski 2003). For the subsequent passages, at 7 dpi, the aerial part of one of the two plants in each lineage was collected. If the two plants presented symptoms, the plant to collect was chosen randomly. If only one presented symptoms, this one was collected. In both cases, the inoculated leaf was removed, and a sap was prepared with 300 mg of symptomatic leaf tissue in 400 μl of inoculation buffer if the infected tissue was from \textit{C. annuum} and 150 mg of infected tissue in 1 ml of inoculation buffer for the other host species, so that each infection was started with similar amounts of viral RNA. For each lineage, two plants were then inoculated on one leaf with 5 μl of sap. Fifteen serial passages were performed. One of the Ca lineages was lost during experimental evolution.

The experimental procedure removes the natural vector of TEV (aphids) and thus represents a simplified version of the virus life cycle. This choice was made because using aphid transmission would have greatly reduced the number of plants we could have infected and consequently the replication level of and within evolutionary history. The evolutionary implications of this choice are discussed below.

**Inf ectivity and Virulence Measurement**

After the 15th passage, infected tissue was collected from each lineage and the viral RNA content measured by RT-qPCR (see Materials and Methods). The obtained quantification was used to prepare saps of equal viral RNA concentration. Each of these saps was mechanically inoculated (5 μl of sap on one leaf) on three plants of each of the four host species. This way, we had a complete crossed design, fully replicated three times. For practical reasons, the inoculation was spread over four days, with the replicate lineages within an evolutionary history split between the days. Additionally, each day three plants of each species were inoculated with inoculation buffer as nonvirus controls, and another three plants were inoculated with a sap (at the same concentration of viral RNA as the evolved lineages) made with the TEV stock used to start the experiment and representing the ancestor for all the evolved lineages. Before inoculation, the aerial part of each plant was measured (from the basis of the stem to the apex) with a precision of 0.5 cm. At 21 dpi, each plant was checked individually and the presence of symptoms was noted, to then calculate the infectivity. The aerial part was measured with a precision of 0.5 cm and weighted (with a Kern 440-35N balance, Kern and Sohn Gmbh) with a precision of 10 mg. We define virulence as the degree of damage...
caused to a plant by viral infection, and it is negatively correlated with host fitness (Shaner et al. 1992; Sacristán and García-Arenal 2008). We calculated the virulence expressed in size as:

\[ \text{Vir}_{size}(E,H_j) = 1 - \frac{\Delta \text{size}(E,H_j)}{\Delta \text{size} \text{(control)}}, \]

where \( \text{Vir}_{size}(E,H_j) \) is the virulence expressed on size of the ith replicate of evolutionary history \( E \) when inoculated on the jth replicate of host \( H \), and \( \Delta \text{size} \) is the difference in size between the day of infection and 21 dpi. A similar virulence index was obtained from the weight, \( \text{Vir}_{weight}(E,H_j) \). However, \( \Delta \text{weight} \) cannot be calculated directly because it is impossible to weigh the plant before inoculation. We thus established the correlation between weight and size for each species for plants of the same age as the ones we inoculated using an independent cohort of healthy plants reared in the same conditions as the one used for infectivity and virulence measurements. Using the correlation for each species and the size at inoculation, we could estimate the weight at inoculation for each plant and thus estimate \( \Delta \text{weight} \).

Genomic Consensus Sequence Determination in the Evolved Lineages

Total RNA was extracted from infected tissue of the 69 experimentally evolved lineages. The TEV genome was amplified in three overlapping fragments and the sequencing of the amplification products was outsourced (for details, see supplementary material, Supplementary Material online). We obtained the consensus sequence from nucleotide 48 to nucleotide 9,492, that is, 99% of the full genome and 100% of the coding sequence. The average coverage was 2.41. The genomes were assembled, and the mutations were identified using the Staden 2.0.0b7 package.

Our estimates of virulence and infectivity were done at the population level, and we did not explore the variability of these variables within each replicate lineage. For this reason, we also directly sequenced polymerase chain reaction (PCR) amplified virus population cDNAs rather than sequencing multiple clones isolated from the population. This consensus sequencing approach allows for detection of the dominant nucleotide at each base position. When multiple clean sequencing reads showed clearly the presence of two peaks at one position, the lineage was considered to be polymorphic at that position. However, it is impossible to measure the frequency of each allele with this method, and mutations could be present in the virus population at frequencies lower than the detection threshold for a chromatogram. The real within-population diversity is thus higher than the one reported here.

Results

Infectivity and Virulence

The analysis of infectivity and virulence data was performed on a data set containing only the data from lineages that had at least one mutation compared with the initial sequence of the ancestral TEV infectious clone. This reduced the data set from 69 to 53 independent lineages (6–9 independent lineages per evolutionary history).

A nominal logistic model with evolutionary history, host plant, and their interaction was built to analyze their effect on infectivity. The “evolutionary history replication” was nested within the “evolutionary history” factor. This analysis revealed that all factors had a significant effect on infectivity (table 1 and fig. 2). Figure 2 also shows the behavior of the ancestral virus, not included in the previously described nominal logistic model. The infectivity of the ancestral virus is in the mid–low range of infectivities of the derived lineages, except on \( N. \) tabacum where it has an infectivity of one. An analysis of variance (ANOVA) with the same factors as above was performed with the virulence expressed on size and on weight as variables. In this case, the replication within evolutionary history was taken as a random factor. The method used was restricted maximum likelihood. The two indices of virulence, evolutionary history did not have a significant effect, whereas host and the interaction between host and evolutionary history had a significant effect (table 2 and supplementary fig. S1, Supplementary Material online). The significant interaction for the three considered variables indicates that the different evolutionary histories produced distinct infection characteristic in the four hosts. Supplementary fig. S1 (Supplementary Material online) also shows the virulence pattern of the ancestral virus across the four hosts (data not included in the previously described ANOVAs): its virulence is in the mid–high range of those of the derived lineages.

Using the full data set of the derived lineages, we could also ask more specific questions: do we have a signature of host specialization? Does this depend on the specialist/general characteristic of the evolutionary history? To answer these questions, each evolutionary history was classified as “specialist” (one host) or as “generalist” (two hosts in alternation). Additionally, each inoculation was classified as “local” or “foreign”: a local inoculation is an inoculation on a host present during the experimental evolution for this precise lineage (e.g., NbNt is local on \( N. \) benthamiana and \( N. \) tabacum; Nb is local on \( N. \) benthamiana), whereas a foreign inoculation is an inoculation on a host which was not present during the experimental evolution (e.g., NbNt is foreign on \( D. \) stramonium and \( C. \) annuum; Nb is foreign on \( N. \) tabacum, \( D. \) stramonium, and \( C. \) annuum). A nominal logistic model was constructed with “host,” “specialist/general,” and “local/foreign,” and their double and triple interactions as factors to determine the effect on infectivity. The specialist/generalist and all interactions containing it did not have any significant effect. Host, local/foreign, and their interaction had a significant effect (table 3).

### Table 1. Nominal Logistic Model on the Infectivity Data of the Experimentally Evolved Lineages.

<table>
<thead>
<tr>
<th>Factor</th>
<th>df</th>
<th>Likelihood Ratio</th>
<th>( \chi^2 )</th>
<th>( P )</th>
</tr>
</thead>
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<tr>
<td>Evolutionary history</td>
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<td></td>
</tr>
<tr>
<td>Host</td>
<td>3</td>
<td>166.34</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Evolutionary history × host</td>
<td>18</td>
<td>83.01</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Replication (evolutionary history)</td>
<td>46</td>
<td>158.77</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** df, degree of freedom.
and the interaction was due to the fact that on *D. stramonium*, the local lineages had an infectivity that was two times larger than the foreign ones (fig. 3A). This effect was present, to a much lower extent, on *N. tabacum*, whereas on the two other hosts local and foreign lineages had identical infectivity. Therefore, this result indicates a strong local adaptation of lineages that have evolved all or part of the time on *D. stramonium*. An ANOVA with the same factors was then performed to analyze the influence on the two virulence indices. For virulence expressed on size, the statistical results were very similar to those for infectivity (table 4), but the origin of the significant interaction was different (fig. 3B): virulence expressed on size was identical for local and foreign lineages on all hosts except on *C. annuum*, where the local lineages had a higher virulence than the foreign ones. For virulence expressed on weight, the factors host and local/foreign had a significant effect, and no other effect was significant. The local/foreign effect was due to a higher virulence of local lineages, particularly on *D. stramonium* and *C. annuum* (fig. 3C).

Another way of looking at local adaptation, which also allows determining whether a cost of adaptation exists, is to perform analyses by pairs of single-host evolutionary histories. Indeed, with the complete factorial design used, the three variables (infectivity, virulence expressed on size, and virulence expressed on weight) were measured for each pair of single-host evolutionary histories in the two corresponding hosts, and we could perform the classical test for local adaptation (Kawecki and Ebert 2004). For each of the six pairs of single-host evolutionary histories and for each host, the difference between the trait value of the local (or native) evolutionary history and that of the foreign (or non-native) evolutionary history was calculated. If this difference is positive, it indicates local adaptation, whereas if it is negative, it indicates local maladaptation. This analysis assumes that our variables are positively related to fitness, which is the case for infectivity and is arguably the case for virulence (see Introduction and Discussion). For each variable, a distribution of 12 values was obtained, and it was possible to test whether the differences were on average positive. The mean is positive for the three variables but not significantly different from zero for any of them: infectivity (one-tailed t-test: \(P = 0.096\); Wilcoxon rank test: \(P = 0.071\)), virulence expressed on size (one-
tailed t-test: \( P = 0.069 \); Wilcoxon rank test: \( P = 0.17 \), and virulence expressed on weight (one-tailed t-test: \( P = 0.064 \); Wilcoxon rank test: \( P = 0.073 \)). However, the combined Fisher test reveals an overall significant local adaptation (combining one-tailed t-tests: \( P = 0.017 \); combining rank tests: \( P = 0.029 \)). Then, using the same differences, it is possible to evaluate the frequency at which the adaptation in one host comes to a cost of adaptation in the other host. For the six pairs of single-host evolutionary histories, we compared the differences previously calculated in each of the two hosts. For example, we compared the (local—foreign) difference in infectivity of the Nb and Nt lineages when inoculated on N. benthamiana with their difference in infectivity when inoculated on N. tabacum. If the two differences are positive, then we have a case of local adaptation with a cost of adaptation. Of the 18 cases (six pairs \( \times \) three variables), there are seven cases of local adaptation with a cost of adaptation, ten cases of local adaptation of one of the evolutionary histories without cost expressed in the other host, and one case of double maladaptation. Of the ten cases of adaptation without cost, five concern lineages evolved on D. stramonium: the adaptation to D. stramonium seems thus to be compatible with the infection and virulence expression in other hosts. Finally, for three of the six pairs of single-host evolutionary histories, data are available for the corresponding alternate host evolutionary histories. Of the seven cases where a cost of adaptation has been identified, we have data for the corresponding alternate host evolutionary history in four cases. There is no clear cost of being a generalist (which would be expressed as a lower value for the generalist strategy than for the locally adapted specialist strategy in each host) in any of these four cases, confirming the idea that the specialist–generalist trade-off is not present in this experimental system. Figure 4 represents the data for virulence expressed on weight for the (Nt, Ca, NtCa) group of evolutionary histories, as an example of a situation with local adaptation, a cost of adaptation, and no cost for the corresponding generalist.

Consensus Sequences

A total of 107 independent mutations occurring at 91 different loci were identified with a range of 0–6 mutations per independent evolved lineage in the 69 independently evolved lineages (for a graphical representation, see fig. 5 and for a complete list, see supplementary table S1, Supplementary Material online). The transition:transversion ratio was 7.2. Sixty-two mutations were synonymous and 45 nonsynonymous, the biological interpretation hereof is discussed below. Of the 107 mutations observed, 26 (24%) were not unique, and of the 91 polymorphic loci identified, 10 (11%) were affected in multiple independent lineages. All the nonunique mutations were repeated in lineages that shared

<table>
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<tr>
<td>Host</td>
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<td>150.52</td>
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<td></td>
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<tr>
<td>Foreign/local</td>
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<td>7.58</td>
<td>0.0059</td>
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<tr>
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<td>0.41</td>
<td>0.5222</td>
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<tr>
<td>Host ( \times ) S/G</td>
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</tr>
<tr>
<td>F/L ( \times ) S/G</td>
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<td></td>
</tr>
<tr>
<td>Host ( \times ) F/L ( \times ) S/G</td>
<td>3</td>
<td>2.53</td>
<td>0.4704</td>
<td></td>
</tr>
</tbody>
</table>

Note.—df, degree of freedom; F/L, foreign/local; S/G, specialist/generalist.

Table 3. Effect of the Type of Evolutionary History (specialist or generalist) and of the Type of Infection (foreign or local) on Infectivity.

<table>
<thead>
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<td>2.53</td>
<td>0.4704</td>
<td></td>
</tr>
</tbody>
</table>


Fig. 3. Experimentally evolved local adaptation: infectivity (A), virulence expressed on size (B), and virulence expressed on weight (C) of local (triangles) and foreign (circles) across the four experimental hosts. All values are expressed ±1 SEM.
a host in their evolutionary history, except in one case (A5409G present in one Nb and one NtCa lines). The very large majority (24 of 26) of nonunique mutations were thus specific to one of the hosts used in the experiment.

We then tested whether the regions encoding for each of the 11 mature proteins had a different rate of mutation accumulation and whether this depended on the evolutionary history of the lineage. To do this, we calculated the number of mutations per nucleotide site for each lineage and for each mature protein. A Scheirer-Ray-Hare nonparametric two-way ANOVA (Sokal and Rohlf 1995) was performed with evolutionary history, protein, and their interaction as factors. It showed that the mutation accumulation per nucleotide is not globally different from one evolutionary history to another ($H_{6,689} = 10.81, P = 0.094$), but that mutations are not randomly scattered across mature proteins ($H_{9,689} = 30.80, P < 0.001$) and that this distribution is different from one evolutionary history to another ($H_{54,689} = 103.57, P < 0.001$). Namely, there is
an overrepresentation of mutations in P1 in lineages Ca, Nt, and NtCa, of mutations in HC-Pro in lineages Ds and DsCa, of mutations in P3 in lineages Nb and NbNt, of mutations in CI in lineages NtCa, of mutations in 6K2 in lineages Nb, of mutations in VPg in lineages Ds, and of mutations in Nla-Pro in lineages Ca.

Focusing on individual consensus sequences, it is possible to identify patterns of mutations that suggest a potential relationship between genotype and phenotype. First, among the Nb lineages, there are two mutations repeated three times, and there is no case where both are present in the same consensus sequence. Moreover, one of them (A3013G, non-synonymous K→E) was also found in three independent NbNt lineages, whereas the other one (A5551G, non-synonymous K→E) was not found in any of the NbNt sequence. This suggests that there are at least two mutations that can improve the adaptation to infection and reproduction in *N. benthamiana* and that one (A3013G) is neutral or advantageous, whereas the other one (A5551G) is deleterious in *N. tabacum*. Second, an identical combination of four mutations (A3013G, C3816A, A6805G, and G8169A) has been found in lineages Nb8 and NbNt6. Except the mutation A3013G, the other three have not been found in any other lineage besides these two. This could be due to the existence of epistatic interactions in this group of mutations. Third, lineages Ca1 and Ca2 have lost the ability to produce systemic infections when inoculated on *N. tabacum* and on *D. stramonium* and form only local lesions on the inoculated leaf but still produce systemic symptoms on *C. annuum* and *N. benthamiana*. The analysis of their sequence revealed only one synonymous mutation in Ca1 (C4425U in the CI region) and two synonymous mutations in Ca2 (A183G and U8574C in the P1 and CP region, respectively).

The relationship between the mutations and the phenotypic type observed is clearly correlational and speculative for now, and the evolutionary mechanisms suggested by the analysis of the sequences have to and will be directly tested in future site-directed mutagenesis experiments.

**Discussion**

TEV evolved differently when experimentally exposed to different evolutionary histories, as evidenced by the phenotypic traits measured, as well as by the sequence changes observed in the evolved lineages. The ancestral virus and the lineages evolved on *N. tabacum*, the ancestral host, present different patterns of infectivity and virulence. This is likely due to an adaptation to the specific conditions of the experimental evolution and in particular to the mechanical transmission, which totally removes the selection due to the aphid transmission. The transmission mode is actually known to affect viral evolution both at the phenotypic and at the genotypic levels (Wallis et al. 2007; Jerzak et al. 2008). Due to these differences between the ancestors and the Nt lineages, we focused the analysis and the discussion on the comparison of the experimental evolutionary histories between them.

We observe that the viral populations have adapted to the hosts that they encountered during their specific evolutionary histories. The analyses performed on pairs of populations further confirm the pattern of local adaptation. In some systems, local adaptation comes at a cost (Hereford 2009), expressed as a worse performance of the locally adapted populations when measured in a foreign environment. In the case of TEV on the four hosts used, the cost of adaptation is not a general feature, as it appears only in some cases. The results for infectivity and virulence were congruent in direction, albeit not in magnitude, for all the analyses performed.

Another interesting result of our study is the absence of cost of generalism, either when we look at the full data set or when we can contrast the characteristics of one generalist evolutionary history with the ones of the two corresponding specialists. This is probably partly due to the fact that the cost of adaptation is not general and consequently, that the adaptation to two hosts simultaneously does not represent a strong evolutionary constraint. However, what might seem harder to explain is the pattern of reciprocal local adaptation with a cost of adaptation and without any identified cost of generalism (e.g., fig. 4). A pattern of reciprocal local adaptation with a cost of adaptation can be due either to antagonistic pleiotropy or to fixation of mutations beneficial in the local host and neutral in the other one. Indeed, in potyviruses, we can exclude the accumulation of deleterious mutations at loci not expressed in one of the hosts (Kawecki 1994) as the full genome is constantly expressed as a single polyprotein. If the cost of adaptation was due to antagonistic pleiotropy, the generalists should pay a cost. Antagonistic pleiotropy makes impossible the adaptation to two hosts at the same time. If it was due to neutral mutations, they could perform as well as the best lineage in each host. Our experimental results favor the second explanation. However, the previous reasoning is based on phenotypic traits only and the sequence data indicate a low level of parallel evolution (see below), meaning that the adaptation is due to different mutations in replicate lineages of an evolutionary history and between evolutionary histories sharing a host. This weakens considerably the argumentation based on phenotypic traits and knowing whether the mutations obtained are antagonistically pleiotropic would require analyzing them one by one through site-directed mutagenesis of the wild-type TEV genome. Finally, another potential, nonexclusive explanation for the absence of cost of generalism is that the “generalist populations” could actually be a composite of two populations, each one being adapted to one of the hosts. If this was the case, it should be visible on the consensus sequence data with a higher proportion of identified polymorphic sites in generalist than in specialist populations. This is, however, not the case: polymorphic sites represent 24.4% of the sites where a change has been identified in generalist lineages compared with 28.8% in specialist lineages (Fisher’s exact test, $P = 0.763$).

The consensus sequences of the evolved populations revealed common mutations between lineages sharing host species in their evolutionary histories. Such parallel evolution is usually interpreted as the fixation of a mutation with
a beneficial effect (Wood et al. 2005), and in this precise case, the host-specific mutations likely increase the level of adaptation to the particular host species. One potential concern about the experimental protocol is that the removal of the aphid vector from the virus life cycle would relax selection pressures at some loci, for example, in the N-terminal part of HC-Pro known to be involved in aphid transmission (Blanc et al. 1998) and that part of the mutations observed would be due to this. The absence of common mutations between lineages that do not share a host excludes the relaxed selection pressure of aphid transmission as the main evolutionary force shaping sequence during our evolution experiment.

Overall, the level of parallel evolution obtained here is below those in other studies comparing genomes from lineages sharing evolutionary history (e.g., Bull et al. 1997; Martínez-Picado et al. 2000; Wichman et al. 2005; Remold et al. 2008). In particular, the experiment conducted by Remold et al. (2008) presents a number of similarities with the present one: it involves experimental evolution of an RNA virus (VSV) by serial transfers either in one of two cell cultures (human or hamster cells) or in the alternation of the two cell types, followed by full genome sequencing of 12 evolved populations. The authors found that 78% of the observed mutations were not unique and 55% of the polymorphic loci were affected in multiple independent populations. The reasons for the lower level of parallel evolution in our system are likely manifold. First, this level strongly depends on the genetic architecture of adaptation, that is, on the distribution of mutation effects in the organisms and in the environments considered (Chevin et al. 2010), but the shape of these distributions is rarely known and consequently, the role of this factor in determining the level of parallel evolution cannot be evaluated on concrete evolution experiments. Second, the lower level of parallel evolution in our study could be due to the narrower and less controlled bottlenecks between passages: we controlled the amount of viral RNA inoculated, but the number of virions actually starting the new infection can vary considerably (Zwart et al. 2011). In any case, the number of virions starting a new infection represents an extremely small fraction of those produced during the colonization of the host in the previous passage and a much smaller fraction than in the case of serial transfer of virus in cell culture. This reduction in effective population size increases the role of genetic drift and reduces the likelihood of fixation of the same mutation in two replicates of the same evolutionary treatment. Third, populations of plant viruses are highly structured because 1) infection within a leaf progresses only from one cell to adjacent ones, 2) viral exclusion phenomenon are frequent (Dietrich and Maiss 2003; Zwart et al. 2011) and reduce the generation of diversity by recombination, and 3) the order of colonization of the leaves is fixed by the phloem distribution. All these factors generate much more opportunities for local competition and exclusion of alleles, more heterogeneity in selection pressures and result in a much smaller effective population size than a well-mixed cell culture. Finally, the low level of parallel evolution might come from the fact that we performed a passage every week during experimental evolution. One week might represent an insufficient time for the virus to generate genetic diversity and for the selection to act on this diversity. However, first for technical reasons and second owing to the drastic effect of TEV on some of the hosts used in this experiment (see virulence levels on N. benthamiana), it was unrealistic to perform longer passages.

For the analysis of consensus sequences, we decided not to interpret the synonymous/nonsynonymous characteristic of the mutations as neutral/nonneutral. Traditionally, synonymous mutations have been considered neutral because they do not induce amino acid changes, and their proportion relative to the nonsynonymous ones has been used to deduce the evolutionary forces at work. However, there is accumulating evidence that synonymous mutations are not always neutral in DNA organisms (Kimchi-Sarfaty et al. 2007; Amorós-Moya et al. 2010; Plotkin and Kudla 2011), and the equivalence of synonymous with neutral is likely to be even weaker in RNA viruses because tridimensional structure is a determinant feature of RNA molecules and it is largely dependent on the sequence itself. The RNA-based genome can thus itself potentially be a target of selection because coding regions serve additional functions other than determining the amino acid sequence in protein. These additional functions include encapsidation or serving as a target of silencing (Cuevas et al. 2012). In TEV, it has been shown that certain synonymous mutations had deleterious effect on the virus multiplication (Carrasco, de la Iglesia, et al. 2007), and the present study provides a striking example of nonneutrality of synonymous mutations with the two lineages which lost the capacity to produce systemic infection on certain hosts and only carry synonymous mutations.

Our experiment, using an in vivo system and a larger replication of and within evolutionary histories, points in the same direction as previous experimental evolution approaches on other pathogens. The generality of the high potential of adaptation of pathogens to new to them at the evolutionary scale considered, indicates that emerging diseases caused by a diversity of organisms actually represent an important threat. The most efficient policies to limit them are preventive and should target the steps prior to adaptation: limit the flow of pathogens toward new host species and avoid generating evolutionary situations favorable to adaptation to a new host.

**Supplementary Material**

Supplementary material, table S1, and figure S1 are available at [Molecular Biology and Evolution](http://www.mbe.oxfordjournals.org/).

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