# Effect of host species on the topography of fitness landscape for a plant RNA virus

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Running Head: empirical fitness landscapes

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## ABSTRACT

**Adaptive fitness landscapes are a fundamental concept in evolutionary biology that relate the genotype of individuals with their fitness. At the end, the evolutionary fate of evolving populations depends on the topography of the landscape, that is, the number of accessible mutational pathways and of possible fitness peaks (*i.e*, adaptive solutions). For long time, fitness landscapes were only theoretical constructions due to a lack of precise information on the mapping between genotypes and phenotypes. In recent years, however, efforts have been devoted to characterize the properties of empirical fitness landscapes for individual proteins or for microbes adapting to artificial environments. In a previous study, we had characterized the properties of the empirical fitness landscape defined by the first five mutations fixed during adaptation of tobacco etch potyvirus (TEV) to a new experimental host, *Arabidopsis thaliana*. Here we evaluate the topography of this landscape in the ancestral host *Nicotiana tabacum*. Comparing the topographies of the landscape in the two hosts, we found that some features remain similar, such as the existence of fitness holes and the prevalence of epistasis, including cases of sign and of reciprocal sign that create rugged, uncorrelated and highly random topographies. However, we also observed significant differences in the fine-grained details among both landscapes due to changes in the fitness and epistatic interactions of some genotypes. Our results support the idea that not only fitness tradeoffs between hosts but also topographical incongruences among fitness landscapes in alternative hosts may contribute to virus specialization.**


## IMPORTANCE

**Despite its importance for understanding virus' evolutionary dynamics, very little is known about the topography of virus adaptive fitness landscapes and even less is known about the effect that different host species and environmental conditions may have of this topography. To bring this gap, we have evaluated the topography of a small fitness landscape formed by all genotypes that result from every possible combination of the five mutations fixed during adaptation of TEV to its novel host *A. thaliana*. To assess the effect that host species may have on this topography, we evaluated the fitness of every genotype both in the ancestral and novel hosts. We found both landscapes share some macroscopic properties such as the existence of holes and being highly rugged and uncorrelated, yet they differ in microscopic details due to changes in the magnitude and sign of fitness and epistatic effects.**

The effect of mutations can be influenced by their interactions with other mutations, with the environment or both. Epistatic interactions among genetic loci determine the ruggedness of fitness landscapes. In the absence of epistasis, landscapes are single-peaked and smooth (1). For such simple landscapes, predicting the result of evolution is an easy task. By contrast, epistatic interactions create curvature in the landscape and, if being of the sign or reciprocal sign types, they create multiple peaks separated by low fitness valleys (1 - 3). The reproducibility of evolution, and therefore our ability to predict its outcome, in such complex landscapes diminishes as the number of possible peaks, that is the ruggedness of the landscape, increases. Therefore, epistasis strongly determines the pace, reproducibility and predictability of adaptive walks on fitness landscapes.

Mutations do not only interact among them in determining fitness; mutations also interact with the external environment, making the phenotypes plastic (4, 5). In practical terms, in the case of viruses the environment mostly reduces to the host, although environmental factors such as temperature, or the presence of other coinfecting viruses or cellular pathogens may affect the replication of viruses as well. Not all potential hosts in the host range (different species or genotypes from the same species) of a virus are equally susceptible to infection, and it is generally assumed that a tight matching may exist between host-genotypes and virus-genotypes to allow a virus to successfully infect a host (6). Indeed, substantial amount of data supports the idea that by evolving into a single host species or genotype viruses become specialists (7 - 10) whereas by evolving in multiple host species, the result may be no-cost generalists (7, 11 - 13).

Furthermore, epistasis and phenotypic plasticity mutually interact in a very intricate manner (14 - 16). The evolutionary consequences of these interactions are important.

81 For example, by changing the pattern of epistasis it is possible to access mutational
82 pathways that may be maladaptive in one environment but not so in another (17 - 20).
83 Understanding the roles that environmental changes and landscape topology have in the
84 number and nature of adaptive pathways would allow predicting the potential avenues
85 of future evolution. Despite this importance, how environmental heterogeneity affects
86 the topography of fitness landscapes is still poorly understood, and only few recent
87 studies have started to tackled this problem; mostly in the context of the evolution of
88 antibiotic resistances (20 - 23) or during experimental adaptation of *Escherichia coli* to
89 an artificial glucose-limited environment (24).

90 The aim of the present study is to explore the effect of environmental changes on
91 the topography of an empirical fitness landscape in a biologically relevant context: an
92 RNA virus and its eukaryotic multicellular hosts. Previously, we constructed the $2^5$ =
93 32 genotypes that comprise all possible combinations of the first five mutations (Table
94 1) fixed by *Tobacco etch virus* (TEV; genus *Potyvirus*, family *Potyviridae*) during
95 experimental evolution on a novel host, *A. thaliana* (25). In this previous work, we
96 evaluated the topography of this fitness landscape in the novel host (26), showing it was
97 rugged and with holes created by the existence of lethal genotype. We also showed that
98 higher-order epistasis, that is, interactions between more than pairs of mutations
99 contributed in a significant manner to the architecture of fitness (26). Here we follow
100 up with this study by evaluating the fitness of all the 32 genotypes in the ancestral host,
101 *N. tabacum*. Comparing both fitness landscapes, we found that some features remain
102 similar among hosts, such as the existence of lethal genotypes and the prevalence of
103 epistasis that create a highly rugged topography. However, we also observed significant
104 differences in the fine grained details among both landscapes due to host-specific
105 effects both in fitness and epistasis.

106

107 **MATERIALS AND METHODS**

108 **Generation of viral genotypes.** All 32 TEV mutant genotypes used in this study were
109 constructed by successive rounds of site-directed mutagenesis starting from the template
110 plasmid pMTEV that contains a full copy of the genome of a wildtype TEV isolated
111 from tobacco (GenBank accession DQ986288) (27), using mutagenic primers with
112 specific single-nucleotide mismatches (26) and Phusion® High-Fidelity DNA
113 Polymerase (Finnzymes). PCR mutagenesis profile consisted of 30 s denaturation at 98
114 ºC, followed by 30 cycles of 10 s at 98 ºC, 30 s at 60 ºC, and 3 min at 72 ºC, ending
115 with 10 min elongation at 72 ºC. Next, the PCR-mutagenesis products were incubated
116 with *Dpn*I (Fermentas) for 2 h at 37 ºC to digest the methylated parental DNA template.
117 *E. coli* DH5α electrocompetent cells were transformed with 2 µl of these reactions
118 products and plated out on LB agar supplemented with 100 µg/ml ampicillin. Bacterial
119 colonies were inoculated in 8 ml LB-ampicillin liquid medium and grown for 16 h in an
120 orbital shaker (37 °C, 225 rpm). Plasmid preparations were done using Pure Yield™
121 Plasmid Maxiprep System (Promega) and following the manufacturer's instructions.
122 Incorporation of mutation was confirmed by sequencing a ca. 800 bp fragment
123 circumventing the mutagenized nucleotide. The plasmid DNA was *Bgl*II linearized and
124 *in vitro* transcribed using mMESSAGE mMACHINE® SP6 Kit (Ambion) in order to
125 obtain infectious RNA of each virus genotype (28).

126 **Plants inoculation.** *N. tabacum* L. cv. Xanthi *NN* plants were used for production
127 of a large stock of virus particles from each of the 32 genotypes. Batches of eight-week
128 old *N. tabacum* plants were inoculated with 5 µg of RNA of each viral genotype by
129 abrasion of the third true leaf. Ten days post-inoculation (dpi), the whole infected
130 plants were collected and pooled for each virus genotype. Next, plant tissue was frozen

131 with liquid $N_2$, homogenized using mortar and pestle and aliquoted in 1.5 ml tubes.
132 Saps were prepared by adding 1 ml of 50 mM potassium phosphate buffer (pH 8.0) per
133 gram of homogenized plant tissue, centrifuged at 4 ºC and 10000 g for 10 min and the
134 upper liquid phase taken and mixed with 10% Carborundum (w/v).

135 These stocks were used to mechanically inoculate either between 3 and 31 (median
136 12) *A. thaliana* L. ecotype L*er*-0 plants, at growth stage 3.5 according to Boyes scale
137 (29), or 6 4-weeks old *N. tabacum* plants. Plants were maintained in a Biosafety Level-
138 2 greenhouse at 25 ºC and 16 h light period. Infection status was determined by one
139 step RT-PCR after 21 dpi for *A. thaliana*.

140 Lethal genotypes and failed experiments produce the same result: a lack of
141 infection. To deal with this possible source of error, we proceeded as described
142 elsewhere (5, 26, 28). In short, we estimated the probability of failing an inoculation
143 experiment using RNA transcripts from wildtype pMTEV using a large number of
144 plants. Then, using this probability, we applied the Bernoulli probability distribution to
145 evaluate the likelihood of failing all inoculation experiments after a given number of
146 trials. In all cases, this probability was $< 0.01$.

147 **Virus genomic RNA purification and quantification of viral load.** RNA
148 extraction from 100 mg of tissue per plant was performed using Agilent Plant RNA
149 Mini Kit (Agilent Technologies) following manufacturer's instructions. The
150 concentration of total plant RNA extracts was adjusted to 100 ng/µl for each sample and
151 the quantification of viral load was done with absolute real time RT-qPCR using
152 standard curves (30). Standard curves were constructed using 10 serial dilutions of
153 TEV genome produced as described above and diluted in total plant RNA obtained from
154 healthy tobacco or arabidopsis plants, treated like all other plants in the experiment.
155 Quantification amplifications were done in 20 µl volume using an ABI StepOnePlus™

156 Real-Time PCR System (Applied Biosystems) with the GoTaq® 1-sStep RT-qPCR
157 System (Promega) as follows: RT phase consisted of 15 min at 37 ºC and 10 min at 95
158 ºC; PCR phase consisted of 40 cycles of 10 s at 95 ºC, 34 s at 60 ºC and 30 s at 72 ºC;
159 final phase consisted of 15 s at 95 ºC, 1 min at 60 ºC and 15 s at 95 ºC. Amplifications
160 were performed in 96-well plates, each plate containing the RNA samples necessary to
161 build the corresponding standard curve; quantifications were performed in triplicate for
162 each sample in different plaques. Quantification results were examined using StepOne
163 software v. 2.2.2 (Applied Biosystems)

164 **Fitness determinations and statistical analyses.** Total RNA was extracted and
165 virus accumulation was quantified by RT-qPCR as described above and detailed in (30).
166 Virus accumulation (pg of TEV RNA per 100 ng of total plant RNA) was quantified at *t*
167 = 21 dpi for *A. thaliana* infected plants and *t* = 5 for *N. tabacum* infected plants to
168 ensure viral populations were at exponential growth in both cases (TEV reaches a quasi-
169 stationary plateau faster in *N. tabacum* than in *A. thaliana*). These values were then
170 used to compute the fitness of the mutant genotypes relative to the wildtype genotype
171 on each host species using the expression $W = \sqrt[t]{R_t/R_0}$, where $R_0$ and $R_t$ are the ratios
172 of accumulations estimated for the mutant and reference viruses, respectively, at
173 inoculation and after *t* days of growth (28).

174 A generalized linear model (GLM) was fitted to the fitness data. The model
175 incorporated three random factors: host species (*H*), virus genotype (*G*) and plant (*P*),
176 which represents the unit of biological replication (different individual plants from host
177 species *H* infected with viral genotype *G*). *H* and *G* were considered as orthogonal
178 factors whereas *P* was nested within the interaction of the *H* and *G*. The model
179 equation reads:

180 $$W_{ijkl} = \mu + G_i + H_j + (G \times H)_{ij} + P(G \times H)_{ijk} + \xi_{ijkl}, \qquad \text{Eq. 1}$$

where $\mu$ is the grand mean value and $\xi_{ijkl}$ is the error associated with individual measure $l$ (estimated from the technical replicates of the RT-qPCR reactions). The statistical significance of each factor was evaluated using a likelihood ratio test (*LRT*) that asymptotically follows a $\chi^2$ distribution. The magnitude of effects was evaluated using the $\eta_P^2$ statistic, the ratio of variance explained by the effect while controlling for the other effects. Effects with $\eta_P^2 \geq 0.25$ are considered as large. Variance components were estimated by maximum likelihood. Statistical analyses were performed with IBM SPSS software version 23.

**Representation of fitness landscapes.** A simple way to represent fitness landscapes is in the form of a graph where each node corresponds to a specific genotype. Instead of representing genotypes in terms of nucleotides or amino acids, one can only indicate whether wildtype or mutant alleles are present at a given site, *i.e.* the possible entries at each site are either ○ or ●, respectively, giving rise to a binary graph. With this notation, wildtype TEV is represented as ○○○○○ while the *Arabidopsis*-adapted isolate is represented as ●●●●●. Edges in the binary graph represent mutational steps of size one, that is, connecting genotypes that only differ in one allele.

**Evaluation of landscapes ruggedness.** The ruggedness of the landscape on the two hosts was evaluated using three different approaches: (*i*) the mean slope to roughness ratio, $\theta$ (31), (*ii*) the correlation between neighbor's fitness, $\rho$, (32) and (*iii*) the frequency of different types of epistatic interactions. $\theta$ measures how much the slope of a given peak spikes out from the average surface in which it exists. A value of $\theta >> 1$ means that a peak emerges from an otherwise flat surface, similar to a Mount Fuji landscape; by contrast, a value of $\theta \leq 1$ would indicate that the peak's slope does not differ substantially from the background surface, that is, it is surrounded by many small peaks of similar slope (31). $\rho$ measures the similarity between the fitness of

206 genotypes that occupy nearby positions in the landscape. If this correlation is perfect ($\rho$
207 $\approx$ 1), then the landscape is absolutely smooth; as epistasis becomes more and more
208 prevalent, then this correlation is reduced, and $\rho < 0$ for the case of sign and reciprocal
209 sign epistasis (Fig. 1) (32).

210 In terms of their effect on landscape topography, four different types of epistatic
211 interactions can be defined (Fig. 1). If magnitude epistasis exists, the fitness of the
212 double mutant is different from the multiplicative expectation (see below for a
213 mathematical definition of this condition). In the example shown in Fig. 1b, the
214 observed fitness of the double mutant is larger than expected (positive epistasis); in the
215 cases of no epistasis (Fig. 1a) or of magnitude epistasis (Fig. 1b), the effects of both
216 mutations are unconditionally beneficial. If the effect of one of the mutations is
217 conditionally beneficial (*i.e.*, beneficial in one genetic background but deleterious in
218 another), then we are in the situation of sign epistasis (Fig. 1c). Finally, if both
219 mutations are deleterious by themselves, but beneficial when combined, we are in the
220 situation of reciprocal sign epistasis (Fig. 1d). The more common the cases of sign and
221 reciprocal sign epistasis, the more rugged the landscape.

222 The graphical representation of the two landscapes and the estimation of the
223 above parameters describing their topography were obtained using the MAGELLAN
224 webserver (33).

225 **Computation of epistasis.** The magnitude of epistasis among mutations $i$ and $j$
226 was calculated as $\varepsilon_{ij} = W_{00}W_{ij} - W_{i0}W_{0j}$, where $W_{i0}$ and $W_{0j}$ are the relative fitnesses
227 of genotypes carrying each single mutation, $W_{ij}$ is the relative fitness of the double
228 mutant, $W_{00}$ is the fitness of the wildtype (34, 35). The second term on the right-hand
229 side of the equation corresponds to the expected fitness which, under the hypothesis of
230 multiplicative independent effects, equals the observed fitness, resulting in $\varepsilon_{ij} = 0$.

Deviations from the null hypothesis indicate antagonistic ($\varepsilon_{ij} > 0$) and synergistic ($\varepsilon_{ij} < 0$) epistasis, respectively. For genotypes containing more than two mutations, a very similar equation can be used: $\varepsilon_{i(k)} = W_{00}W_{i(k)} - W_iW_{(k)}$ but in this case $W_{(k)}$ corresponds to the fitness of the genotype containing $k$ mutations into which mutation $i$ has been introduced and $\varepsilon_{i(k)}$ is the epistasis between mutation $i$ and the genetic background containing the $k$ other mutations. For example, genotype ●○●●○ could be constructed in three ways: inserting mutation ○○○●○ into genetic background ●○●○○, inserting mutation ○○●○○ into genetic background ●○○●○, and by inserting mutation ●○○○○ into ○○●●○, meaning that we can test for three cases of epistasis in this genotype. This decomposition of interactions generates 75 possible genetic combinations for which epistasis was tested. Following the mathematical conditions given in (36), we evaluated whether those cases for which we estimated a significant epistasis coefficient also corresponded to sign or reciprocal sign epistasis.

Higher-order epistasis were also evaluated using the Walsh coefficients, as proposed in (37), using the MAGELLAN webserver (33). Walsh coefficients have their equivalent in classic population genetics (37): (*i*) zero-order Walsh coefficients represent the mean fitness across all genotypes; (*ii*) first-order Walsh coefficients are equivalent to the selection coefficients, which represent the fitness effects of single mutations; (*iii*) second-order Walsh coefficients are equivalent to pairwise epistatic coefficients, $\varepsilon_{ij}$; and (*iv*) higher-order Walsh coefficients are thus equivalent to higher-order epistatic interactions among the > 2 mutations present in a genotype. In the case of multiplicative fitness landscapes, all Walsh coefficients for order ≥ 2 are equal to zero and, thus, the landscape is smooth. In contrast, ruggedness is maximal in a fully random landscape, with many local peaks representing all types of epistatic interactions, and thus, the mean squared epistatic coefficients increase exponentially with order.

256 Real fitness landscapes shall lie between these two extremes, neither smooth nor
257 maximally rugged (37).
258
259 **RESULTS**
260 **Landscapes topographies and first descriptive statistics.** Fig. 2 shows both
261 estimated landscapes and Table 2 contains some statistics describing their topographies.
262 Defining a peak as a genotype such that all their neighbors have lower fitness, in *A.*
263 *thaliana*, these 32 genotypes define a landscape with two peaks (genotypes ○●○○●
264 and ○○●●○) of different height (26), whereas four peaks are defined in the ancestral
265 host *N. tabacum* (genotypes ●○○○○, ○●○○●, ○○○●●, and ●●●●○). The
266 ruggedness of the landscape can be evaluated using several different measures (Table
267 2). For instance, the ratio between mean slope and roughness, $\theta$, (31) took similar
268 values for both hosts, and in both cases values were greater than 1, indicating that the
269 landscapes are rugged in relationship to the average slope of the peaks. The amount and
270 type of epistasis also describe the topography of a landscape. A recently proposed
271 measure of epistasis is the correlation between fitness effects of a given genotype and
272 all their one-step neighbors, $\rho$ (32). In our case, both $\rho$ values are positive and small,
273 close to zero, suggesting the existence of many cases of magnitude epistasis. Another
274 very intuitive measure of the ruggedness of a landscape is to compute the frequency of
275 each type of epistatic interactions among all possible pairs of mutations, for a smooth
276 landscape, the fraction of multiplicative interactions should be maximal; as the
277 ruggedness of the landscape increases, cases of sign and of reciprocal sign epistasis
278 should become more common (2, 36). Table 2 indicates that most mutations interacted
279 epistatically in both hosts, with magnitude epistasis being the most common type of
280 interaction in both landscapes. Sign epistasis was the second most common type of

epistasis in *A. thaliana* while reciprocal sign epistasis was so for the ancestral host *N. tabacum*. All together, these result suggest that the landscape defined by these five mutations is more rugged in the ancestral host than in the new one. We will further expand these results in the next sections.

**Fitness correlations and antagonistic pleiotropy among hosts.** To further explore the relationship between the topographies of both landscape shown in Fig. 2, we have evaluated the similarity in the fitness effects estimated for each genotype in each host species (Fig. 3a). Fitness values are significantly correlated among the two landscapes (Pearson's $r = 0.891$, 30 d.f., $P < 0.001$). However, this correlation is entirely driven by the existence of a group of genotypes that are lethal in both hosts. If these genotypes are removed from the analysis the correlation is not significant anymore ($r = 0.338$, 20 d.f., $P = 0.124$). The dashed lines in Fig. 3a represent the relative fitness of the wildtype TEV in both hosts. These lines divide the fitness space in four regions, each region corresponding to the genotypes with fitness values greater or smaller than the wildtype (○○○○○) in each hosts. Twelve genotypes had fitness values greater than wildtype in both hosts and thus are unconditionally beneficial. By contrast, 10 genotypes were unconditionally deleterious, with fitness values smaller than wildtype in both hosts. Nine of them were lethal in both hosts and genotype ○○●○○ was lethal in *A. thaliana* but only slightly deleterious (–0.5% effect) in *N. tabacum*. Together, these 22 genotypes occupying the upper-right and lower-left quadrants of Fig. 3a account for the above correlation. Cases in the other two quadrants are more interesting as they represent examples of antagonistic pleiotropy: genotypes beneficial in one host that are deleterious in the alternative one. Genotype ●○○○○ was beneficial in *N. tabacum* but it was deleterious in *A. thaliana*. Eight genotypes had fitness values greater than the wildtype in *A. thaliana* but were deleterious in the

original host. Given their low fitness in the ancestral host, most likely these genotypes were generated and selected during the process of adaptation to the new host.

Prior to any further statistical analyses, fitness data were checked for violation of the assumptions of normality and homocedasticity of variances. We found that data were not normally distributed (one-sample Kolmogorov-Smirnov test: $D = 0.248$, $P < 0.001$) nor variances were homogeneous among groups (Levene test: $F_{229,399} = 13.980$, $P < 0.001$). The GLM described in Eq. 1, with a gamma distribution and a log-link function (chosen because it had the minimal Bayes information criterion among a set of alternatives tested), was fitted to the fitness data to evaluate the relative contribution of genotypes and host species to the observed variability in fitness. Table 3 shows the results of this analysis. Overall, highly significant differences exist among the 32 genotypes ($P < 0.001$), which largely contribute to the observed differences in fitness ($\eta_P^2 = 0.860$). The percentage of total variance explained by true genetic differences among viral genotypes is as large as 77.8%. The net contribution of host species to viral fitness is also significant ($P = 0.037$), although the magnitude of the effect is very small ($\eta_P^2 = 0.006$; variance explained by host: only 0.2%) and consequently the statistical power associated to this test is too low to make the result reliable. However, a highly significant effect ($P < 0.001$) of host species that depends on each genotype exists, being the magnitude of this interaction effect also large ($\eta_P^2 = 0.500$; variance explained by interaction: 16.7%). The fact that the interaction between viral genotypes and host species contributes to fitness in a much larger extent than host species itself has an important consequence: the two landscapes differ in fine-grained details more than they do in the coarse-grained details. Finally, differences among plants of each host species inoculated with the same viral genotype are also significant ($P < 0.001$) and of a

magnitude comparable to that of the main virus effect ($\eta_P^2$ = 0.848), but explains a relatively minor fraction of the total observed variance (4.2%).

**Differences in epistasis and landscape ruggedness among hosts.** Next, we explored the congruency between epistasis values and types across host species for all genotypes carrying two or more mutations. Computing epistasis between pairs of mutations is straightforward, however, for genotypes carrying more than two mutations, the computation becomes slightly more complicated. For example, for a triple mutant, we must consider the three different cases in which each single mutation is introduced into the corresponding complementary double-mutant genotypes (see Materials and Methods for an example). By doing so, we have to analyze a total of 75 different possibilities (26). Fig. 3b shows these data and illustrates the existence of a significant correlation between epistasis coefficients measured in both hosts ($r$ = 0.718, 73 d.f., $P$ < 0.001). However, a substantial number of interactions do not fit the diagonal expected under the hypothesis of no host effect on epistasis. Most of these cases, 10, had negative epistasis in *N. tabacum* that changed into multiplicative effects or even positive epistasis in *A. thaliana*.

Genetic interactions in *A. thaliana* can be classified as follows: 40 cases are multiplicative, 26 of magnitude epistasis, four of sign epistasis, and five of reciprocal sign epistasis (26) (Table 4). In *N. tabacum* the counts per category are: 46 cases of multiplicative interactions, 11 of magnitude epistasis, seven of sign epistasis, and 11 of reciprocal sign epistasis. The distribution of counts per categories is significantly different among hosts ($\chi^2$ = 10.050, 3 d.f., $P$ = 0.018), with an excess of cases of sign and reciprocal sign in *N. tabacum*. The epistasis-transition matrix (Table 4) shows the effect that experimental adaptation to *A. thaliana* had on the different types of epistasis. Most of the interactions remained of the same type on both hosts (65.3%, Binomial test

355 $P$ = 0.011), mainly due to the congruency in the number of multiplicative cases.
356 Interestingly, among those that changed the type of epistasis, 57.5% did so in the
357 direction of reducing the ruggedness of the landscape (*e.g.*, from sign or reciprocal sign
358 to magnitude) in *A. thaliana*.

359 Seeking for a mechanistic understanding of these changes in the patterns of
360 epistasis, we will focus in pairwise interactions due to their simplicity. Five
361 combinations of two mutations resulted in a reduction of the landscape's ruggedness in
362 *A. thaliana* compared to *N. tabacum*, in three cases from reciprocal sign epistasis to
363 magnitude epistasis and in one case from sign epistasis to multiplicative effects.
364 Noteworthy, four out of these five case involved synonymous mutation *P1*/U357C
365 (●○○○○). No obvious explanation can be brought forward to explain why the effect
366 of a synonymous mutation depends so strongly on the presence of mutations in other
367 genes. Another interesting case is nonsynonymous mutations 6K1/T1126M (○○●○○).
368 The 6K1 small peptide is required for viral replication and colocalizes with chloroplast-
369 bounded viral replicase elements 6K2 and NIb at early stages of infection (38). The
370 fitness effects resulting from the interaction between this particular mutation at 6K1 and
371 all other four mutations were always host-dependent. When combined with
372 synonymous mutation *P1*/U357C or with nonsynonymous mutation P3/A999V
373 (○●○○○), interactions changed from sign in *N. tabacum* to multiplicative or to
374 magnitude epistasis in *A. thaliana*, respectively. However, when this mutation was
375 combined with nonsynonymous mutation VPg/L1965F (○○○●○) or with synonymous
376 mutation *NIaPro*/C6906U (○○○○●), it increased ruggedness from multiplicative to
377 sign or to magnitude epistasis, respectively, in the novel host. Again, no obvious
378 mechanism can be brought forward to explain why the effect of this mutation depends
379 on synonymous mutations in other genes. The fitness effect of 6K1/T1126M is

alleviated in presence of mutation P3/A999V in the novel host, suggesting some form of interaction between these two genes (either direct or indirect) not yet detected experimentally (39). The beneficial fitness effect of 6K1/T1126M is potentiated in presence of mutation VPg/L1965F in the novel host, also suggesting that these two proteins may tightly coordinated actions in determining TEV fitness in the novel host. Neither a direct interaction between these two proteins has been confirmed experimentally (39). However, in both cases, an indirect interaction mediated by the CI protein may still be possible (39).

So far, we have focused on pairwise interactions between individual mutations or between a mutation and a group of them. Weinreich *et al*. (37) pointed out that this approach must be misleading as the products of many genes interact in complex manners to determine the fitness of individuals, thus higher-order epistasis must be a fundamental component of the genetic architecture of fitness. Using the Walsh's coefficients approach proposed in (37), we have evaluated the contribution of higher-order epistasis to the two landscapes. Fig. 4 compares the weight of each Walsh's coefficient to the fitness variability observed in both landscapes. The zero-order coefficients represent the mean fitness across all genotypes. In this case, mean fitness is higher in the novel host than in the ancestral one. This is logical, since these genotypes, at least those that may have a real existence in the evolving population, were positively selected in *A. thaliana*. First-order coefficients correspond to selection coefficients of single mutations. In both landscapes, up to four-order interactions contribute in a noticeable manner to the observed pattern of fitness, illustrating the complexity of interactions between genes in determining TEV fitness in both hosts. Interestingly, second-order interactions, that correspond to pairwise epistasis coefficients, seem to be qualitatively more important in *A. thaliana*, while third-order interactions representing

405 the effect of a given mutation on the curvature of the surface defined by two other
406 mutations (*i.e.*, second-order interactions) appear to be more important in *N. tabacum*.
407 The different weight of second- and third-order interactions in each hosts further
408 supports the idea that the landscape was less rugged in the novel host than in the
409 ancestral one. Four-order interactions also appear to be qualitatively more important in
410 *A. thaliana* than in *N. tabacum*. Four-order coefficients reflect the effect that a surface
411 defined by a pair of mutations exerts on the surface defined by another pair of
412 mutations. Unfortunately, we cannot provide and intuitive visualization this numerical
413 result.

414 **Relationship between antagonistic pleiotropy and the sign of epistatic**
415 **interactions.** Pleiotropy and epistasis have strong parallelisms because the effect of an
416 allele depends on the context in both cases: the host species for pleiotropy and the virus'
417 genetic background for epistasis. Indeed, it has been postulated that pleiotropy is a
418 prerequisite for epistasis (3, 40). This dependence is obvious for the case of sign
419 pleiotropy, where mutations with a positive effect in the new host have a negative effect
420 in the primary one (13). Furthermore, in the context of compensatory evolution,
421 antagonistic pleiotropy is a precondition for sign epistasis, because it allows for the
422 negative pleiotropic effects of previously selected mutations to be compensated by
423 additional ones (3). Therefore, it is of interest to test whether the eight genotypes
424 showing evidences of antagonistic pleiotropy (see comments above on Fig. 3a), all of
425 them carrying nonsynonymous mutations 6K1/T1126M, also change the sign of their
426 epistatic interactions in both hosts. Indeed, all eight genotypes show a change in the
427 sign of their epistatic interactions: genotype ○●●○○ from negative to positive and the
428 other seven genotypes from positive to negative. By contrast, among the 18 genotypes
429 not showing evidences of antagonistic pleiotropy, 14 do not change the sign of their

epistatic interactions among hosts species and four do change (three from negative to positive and only one from positive to negative). A Fisher's exact test confirms that changes in the sign of epistasis are significantly enriched among genotypes showing antagonistic pleiotropy compared with genotypes that did not showed it ($P < 0.001$).

## DISCUSSION

The results described above clearly illustrate that changes in host species result in perturbations in the topography of the fitness landscape of an RNA virus. The five mutations fixed during experimental evolution of TEV in the novel host *A. thaliana*, conformed a landscape in the original host, *N. tabacum*, that was significantly more rugged that the landscape in the novel host. Differences among both landscapes, however, were local rather than global, with particular genotypes changing their relative height in the landscape and resulting in different patterns of epistatic interactions with their neighbors. This dependence of the topography of the fitness landscape on the host supports the notion of dynamic landscapes (17) or seascapes (19) rather than of static ones. Nonetheless, both landscapes shared common features, such as the existence of fitness holes due to unconditionally lethal genotypes or the presence of pervasive epistatic interactions. The topography of both empirical landscapes match pretty well with the expectations from a random uncorrelated landscape; lying somewhere between the extreme case of the House-of-Cards model (31, 41), in which the fitness of each genotype is absolutely independent on the fitness of the other genotypes, and the less radical case of the rough Mount Fuji model (31, 42), which combines properties of both the House-of-Cards and of a purely multiplicative landscape.

Antagonistic pleiotropic fitness effects have been widely reported for RNA viruses adapting to different hosts and are generally accepted as the main cause of fitness

455 tradeoffs among hosts that drive virus specialization to novel hosts (reviewed in (43)).
456 Here we have found that ~26% of genotypes have a pleiotropic fitness effect, with all
457 but one of these cases corresponding to genotypes beneficial in the novel host but
458 deleterious in the ancestral one. These results further stress the importance of
459 antagonistic pleiotropy in driving adaptation to local new host at the cost of a reduced
460 fitness in the ancestral one. Other authors, however, consider that fitness tradeoffs have
461 been overrated as the mechanism explaining virus' host specialization (44, 45). Indeed,
462 it has been proposed that incongruent fitness landscapes may be a better explanation for
463 the evolution of specialist viruses infecting alternative hosts (45). Our results show that
464 these two hypotheses can be conciliated: some genotypes represent clear examples of
465 antagonistic pleiotropy while both landscapes are incongruent in some particular details.
466 Indeed, both hypotheses are not mutually exclusive as antagonistic pleiotropy largely
467 contributes to the incongruence among landscapes.

468 We have also found that antagonistic pleiotropy in host usage and epistasis at the
469 genomic level go hand by hand, thus corresponding to a situation defined as epistatic
470 pleiotropy (13). Indeed, we have previously reported a similar result when analyzed the
471 fitness and epistatic interactions of a larger collection of random mutations on TEV
472 genome (16). Epistatic pleiotropy has two important implications. Firstly, unlike either
473 sign or magnitude pleiotropy in the absence of epistasis, epistatic pleiotropy allows for
474 the evolution of either specialist or no-cost generalist viruses, depending on the virus
475 population's host. Secondly, and very important to limit the emergence of new viruses,
476 when epistasis is in the form of reciprocal sign epistasis, the ruggedness of the adaptive
477 landscapes diminishes the ability of viral populations to escape from specialism to a
478 situation of no-cost generalism. A long history of evolution in the primary host may
479 result in an adaptive walk towards a host-specific fitness peak involving most, if not all,

480 viral loci. Such a population could find itself many mutational steps away from
481 reaching a generalist peak.

482 In recent years evolutionary biologists have started to tackle the topography of
483 fitness landscapes from an empirical perspective (reviewed in (1)). Unfortunately, the
484 amount of information about fitness landscapes is still very limited. Empirical fitness
485 landscapes have been thoroughly explored only for another virus, HIV-1, for mutations
486 allowing access to alternative cell surface chemokine co-receptor (46 - 48), and for
487 adaptation to different antiviral drugs (49). In both cases, ruggedness has been proved
488 to be common due to the pervasiveness of epistasis. In the latter case, results suggested
489 that the coarse-grained details of the topography were only weakly dependent on
490 environmental conditions, in this case the presence of different antiretroviral drugs (49).
491 Our results are in good agreement with these previous findings.

492 How can viral population reach the global fitness maximum in such a highly rugged
493 landscape and not be trapped in suboptimal fitness peaks? Here we have shown that by
494 changing the host species the landscape has been flattened off, facilitating the access to
495 certain peaks that otherwise may remain inaccessible in the ancestral host. There are
496 other possible mechanisms for efficiently improving fitness in such landscapes that do
497 not necessarily require moving one step at a time. This long-range jumps are known as
498 stochastic tunneling in large populations (50). Recombination is the most obvious
499 mechanism for such tunneling effect as it may combine beneficial mutations into a
500 single genotype. At least for TEV, the recombination rate is on the same ball park than
501 mutation rate (51) and high recombination rates are not rare among (+)ssRNA viruses
502 (52). The typically high mutation rates of RNA viruses, usually on the vicinity of one
503 per genome and replication round (53), combined with their very fast replication rates
504 and large population sizes make likely that a double mutant carrying two beneficial

mutations could be created, thus allowing for the tunneling effect. In the case of TEV, genomic mutation rate is $U = 0.601$ (54). Assuming that only a very minor fraction of all possible individual mutations is beneficial, say only one per genome, the lower bound probability of finding a genome carrying two of such beneficial mutations would be $U_b^2 = (0.601/9539)^2 = 3.97 \cdot 10^{-9}$. From an evolutionary perspective the number that matters is the product $NU_b^2$, where $N$ is the population census size. This product gives the number of individuals in the population that are double mutants. For TEV $N$ strongly varies among hosts but in the case of susceptible *A. thaliana* ecotypes, it is always greater than $10^8$ and it can be as large as $10^{10}$ genomes per plant (55), thus making $NU_b^2$ very likely to be greater than one during the course of most infections.

Some readers may consider as caveats of this study that (*i*) *A. thaliana* is not a natural host of TEV and (*ii*) that all our experiments have been performed in controlled greenhouse conditions that may be optimal for virus replication and accumulation. We do not consider the first to be a real problem as this study, and all previous ones performed with the same experimental pathosystem (25, 26, 30, 55 - 59), deal with the evolutionary determinants and consequences of viral emergence and adaptation to a fully novel host. The second may certainly be an issue to be considered. It is well known that *A. thaliana*, and wild hosts in general, support less replication than crops or hosts grown in greenhouse conditions (60). In this sense, our arguments above for efficient landscape exploration based on stochastic tunneling may not work well in the wild if replication levels are reduced. Therefore, generalizing our findings and conclusions to a natural ecological context may not be straightforward… as it might be the case for almost every experimental evolution study, at least, if not for every laboratory experiment.

As a closing consideration, gathering information on the structure and topology of RNA virus' adaptive landscapes, on their dependence on external factor and on how they modulate virus evolution may be central for developing new antiviral strategies, personalized clinical treatments and predicting and containing emerging diseases of viral etiology.

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TABLE 1 Set of mutations included in this study.

| Label | Mutation | Gene | Amino acid change[a] |
|---|---|---|---|
| ●○○○○ | U357C | *P1* | synonymous |
| ○●○○○ | C3140U | *P3* | A999V |
| ○○●○○ | C3629U | *6K1* | T1162M |
| ○○○●○ | C6037U | *VPg* | L1965F |
| ○○○○● | C6906U | *NIaPro* | synonymous |

[a] Numeration according to the amino acid residue in the polyprotein.

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696

TABLE 2 Summary statistics describing the topography of both landscapes.

| Statistics[a] | *A. thaliana* | *N. tabacum* |
|---|---|---|
| General: | | |
| Peaks | 2 | 4 |
| Sinks | 0 | 0 |
| Epistasis: | | |
| Mean slope to roughness ratio ($\theta$) | 1.902 | 1.697 |
| Correlation between neighbors' fitness ($\rho$) | 0.119 | 0.111 |
| Frequency multiplicative | 0.013 | 0.013 |
| Frequency magnitude | 0.662 | 0.575 |
| Frequency sign | 0.212 | 0.188 |
| Frequency reciprocal sign | 0.113 | 0.225 |

[a] Computed using the MAGELLAN webserver (33).

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TABLE 3 Summary of the GLM model fitted to the data.

| Factor | $LRT$ [a] | $df$ | $P$ | $\eta_P^2$ [b] | $1-\beta$ [c] |
|---|---|---|---|---|---|
| Intercept | 3979.285 | 1 | < 0.001 | 0.982 | 1 |
| *G* | 2397.695 | 31 | < 0.001 | 0.860 | 1 |
| *H* | 4.341 | 1 | 0.037 | 0.006 | 0.183 |
| *G*x*H* | 1344.481 | 20 | < 0.001 | 0.500 | 1 |
| *P*(*G*x*H*) | 1168.930 | 177 | < 0.001 | 0.848 | 1 |

[a] Likelihood ratio test.

[b] Magnitude of effects associated to each model factor.

[c] Statistical power of the corresponding tests.

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TABLE 4 Epistasis transition matrix.

| | *A. thaliana* | | | |
|---|---|---|---|---|
| *N. tabacum* | No | Magnitude | Sign | Reciprocal sign |
| No | 37 | 8 | 1 | 0 |
| Magnitude | 2 | 9 | 0 | 0 |
| Sign | 1 | 4 | 0 | 2 |
| Reciprocal sign | 0 | 5 | 3 | 3 |

699

700 FIG 1 Different types of epistasis between two loci defining the fitness of a genotype.
701 Small letters indicate the wildtype and capital letters the mutant alleles. (a) In case of
702 no epistasis, the fitness of the double mutant ●● results from multiplying the fitness
703 effects of both mutations on the wildtype genetic background (*i.e.*, the fitnesses of
704 genotypes ●○ and ○●). (b) If magnitude epistasis exists, the fitness of the double
705 mutant ●●is different from the multiplicative expectation. In the example, the
706 observed fitness of ●●is larger than expected as a consequence of positive epistasis.
707 Both in the cases of no epistasis or of magnitude epistasis, the effects of mutations ●○
708 and ○● are unconditionally beneficial. (c) If the effect of one of the mutations is
709 conditionally beneficial (*i.e.*, beneficial in one genetic background but deleterious in
710 another), then we are in the situation of sign epistasis. (d) Finally, if both mutations ●○
711 and ○● are deleterious by themselves, but beneficial when combined, we are in the
712 situation of reciprocal sign epistasis.
713
714 FIG 2 Empirical fitness landscapes evaluated for the set of five mutations fixed by TEV
715 during its experimental adaptation to *A. thaliana*. The fitness of the 32 genotypes was
716 evaluated in the novel host (a) and in the original one, *N. tabacum* (b). Each string of
717 dots represents a genotype. Black dots represent a mutation in the corresponding locus,
718 while white dots correspond to the wildtype allele on this locus. Genotypes in a green
719 box correspond to local fitness peaks. Green lines correspond to beneficial mutations,
720 red lines to deleterious mutations and orange lines to neutral changes (in the direction
721 from genotype ○○○○○ to genotype ●●●●●). Graphs generated with the
722 MAGELLAN webserver (33).
723

FIG 3 Fitness values and epistasis coefficients in both hosts. (a) Fitness values estimated for the 32 genotypes shown in Fig. 2 in both hosts. In both hosts, fitness is expressed relative to the wildtype genotype ○○○○○. Dashed lines correspond to the fitness of wildtype on each host. The (0, 0) dot correspond includes the nine cases of unconditionally lethal genotypes. (b) Distribution of epistasis on both hosts. Dashed lines correspond to the case of multiplicative fitness effects (no epistasis). Error bars correspond to ±1 SD.

FIG 4 The Walsh's coefficient of order zero is the mean fitness across all genotypes; fitness values were normalized to make this figure equal to one. First-order and second-order coefficients are analogous to selection coefficients and pairwise epistasis, respectively. Higher order terms are equivalent to epistasis among increasing numbers of mutations. Walsh's coefficients were computed with the MAGELLAN webserver (33).