Host Resistance Selects for Traits Unrelated to Resistance-Breaking That Affect Fitness in a Plant Virus

Aurora Fraile,1 Jean-Michel Hily,1 Israel Pagan,1 Luis F. Pacios,2 and Fernando Garcia-Arenal*1

1Centro de Biotecnología y Genómica de Plantas (UPM-INIA) and E.T.S.I. Agrónomos, Campus de Montegancedo, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain
2Centro de Biotecnología y Genómica de Plantas (UPM-INIA) and E.T.S.I. Montes, Campus de Montegancedo, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain

*Corresponding author: E-mail: fernando.garciaarenal@upm.es.

Abstract

The acquisition by parasites of the capacity to infect resistant host genotypes, that is, resistance-breaking, is predicted to be hindered by across-host fitness trade-offs. All analyses of costs of resistance-breaking in plant viruses have focused on within-host multiplication without considering other fitness components, which may limit understanding of virus evolution. We have reported that host range expansion of tobamoviruses on L-gene resistant pepper genotypes was associated with severe within-host multiplication penalties. Here, we analyze whether resistance-breaking costs might affect virus survival in the environment by comparing tobamovirus pathotypes differing in infectivity on L-gene resistance alleles. We predicted particle stability from structural models, analyzed particle stability in vitro, and quantified virus accumulation in different plant organs and virus survival in the soil. Survival in the soil differed among tobamovirus pathotypes and depended on differential stability of virus particles. Structure model analyses showed that amino acid changes in the virus coat protein (CP) responsible for resistance-breaking affected the strength of the axial interactions among CP subunits in the rod-shaped particle, thus determining its stability and survival. Pathotypes ranked differently for particle stability/survival and for within-host accumulation. Resistance-breaking costs in survival add to, or subtract from, costs in multiplication according to pathotype. Hence, differential pathotype survival should be considered along with differential multiplication to understand the evolution of the virus populations. Results also show that plant resistance, in addition to selecting for resistance-breaking and for decreased multiplication, also selects for changes in survival, a trait unrelated to the host–pathogen interaction that may condition host range expansion.

Key words: virus evolution, host range expansion, gene-for-gene, survival, fitness trade-offs, tobamovirus.

Introduction

Host range is a key property of parasites, conditioning their ecology and epidemiology and, hence, the control of extant infectious diseases and the emergence of new ones (Frank 1996; Woolhouse et al. 2001; Lajeunesse and Forbes 2002; Elena et al. 2009). Consequently, the evolution of host range in parasites has received considerable attention. Although a large host range would provide a parasite with more opportunities for transmission and survival, it has been suggested that differential host-associated selection will favor the evolution of specialist parasites adapted to one or a few related hosts (Woolhouse et al. 2001; Kirchner and Roy 2002; Elena and Sanjuán 2003). The basis for this hypothesis is that increasing the fitness of a parasite in one host will decrease its fitness in another host, resulting in across-host fitness trade-offs, which will hinder host range expansions. A particular case of host range expansion is the overcoming of host resistance, that is, the acquisition by the parasite of the capacity to infect, multiply, and cause disease in host genotypes resistant to other parasite genotypes, which expands the parasite’s host range (Gandon et al. 2002; Tellier and Brown 2007, 2009).

Resistance-breaking has been analyzed in high detail in plant parasites, because breeding crop cultivars resistant to pathogens is an efficient, specific, and environment-friendly strategy for the control of crop diseases. However, the effective life of resistant cultivars is often short due to the appearance and increase in frequency of resistance-breaking genotypes in pathogen populations (Bergelson et al. 2001; McDonald and Linde 2002; García-Arenal and McDonald 2003; Kang et al. 2005; Maule et al. 2007; Sacristán and Garcia-Arenal 2008). Plant–parasite interactions often conform to the gene-for-gene (GFG) model, in which plant resistance genes (R) encode for proteins able to recognize parasite proteins encoded by avirulence (Avr) genes, and R-Avr recognition triggers a defense reaction limiting parasite multiplication to the infection site (Jones and Dangl 2006; Brown and Tellier 2011). Mutations in the Avr gene that impair R-Avr recognition result in resistance-breaking. A key feature of GFG systems is that universal virulence, that is, the capacity to infect all host genotypes, evolves as a result of subsequent resistance-breaking events (Agrawal and Lively 2002). Thus, the evolution of resistance-breaking in GFG systems is a case of host range expansion. GFG models consider that the capacity to overcome a resistance gene would be linked to
a decrease of parasite fitness in susceptible host genotypes, that is, to resistance-breaking costs that are an instance of across-host fitness trade-offs. Theoretical analyses of the evolution of GFG systems have shown that costs of resistance-breaking are necessary, but not sufficient, for the occurrence of stable resistance-avirulence polymorphisms (Brown and Tellier 2011). Because resistance-breaking costs are central to plant–parasite coevolution under the GFG model and may determine the durability of resistance for controlling plant diseases, much effort has been devoted to their experimental detection and quantification. Despite some notable exceptions (e.g., Thrall and Burdon 2003; Huang et al. 2006; Bahri et al. 2009; Montarry et al. 2010), detection of such costs in cellular plant pathogens has been mostly inconclusive (Leach et al. 2001; Sacristán and García-Arenal 2008). On the other hand, definitive evidence for resistance-breaking costs has been reported for several plant–virus systems (Sacristán and García-Arenal 2008; Fraile and García-Arenal 2010). All reports of resistance-breaking costs in plant viruses derive from the analysis of virus multiplication in the infected host, either in single infection or in competition with non-resistance-breaking genotypes (Ayme et al. 2006; Janzac et al. 2010; Poulicard et al. 2010, 2012; Fraile et al. 2011; Ishibashi et al. 2012; Montarry et al. 2012). Other components of viral fitness have not been considered, which may limit our understanding of plant–virus coevolution. As shown for other plant pathogens (Montarry et al. 2010; Pariaud et al. 2012), trade-offs between different fitness components could determine the relationship between resistance-breaking and viral fitness.

The purpose of this work was to deepen our understanding of the relationship between host range expansion and fitness in plant viruses. To this end, we analyzed whether host range expansion into previously resistant host genotypes, which was associated with lower within-host multiplication, was also associated with penalties in another major fitness component, that is, survival.

A well-characterized experimental system was chosen: the tobamoviruses that infect pepper (Capsicum annuum L.) genotypes carrying different resistance alleles at the L locus. Viruses in the genus Tobamovirus are RNA viruses with a single-stranded, monopartite genome encapsidated into rod-shaped particles, and particle structure has been resolved by X-ray crystallography for several tobamovirus species (Namba et al. 1989; Pattanayek and Stubbs 1992; Wang et al. 1997). Pepper-infecting tobamoviruses are transmitted in nature through plant-to-plant contact or, vertically, through the seed. After infected plant tissue degradation, infectious particles can survive for several months in the soil. These represent a major source of primary inoculum for yearly epidemics and the only source if seed health certification programs are implemented (Broadbent 1976; Sakamoto et al. 2008). Pepper-infecting tobamoviruses are controlled by resistance conferred by four alleles at the L locus (Boukema 1980, 1984). The interaction of tobamoviruses and L locus resistance follows a GFG model, with different tobamoviral species and genotypes being classified into separate pathotypes according to their capacity to infect pepper genotypes carrying the various L alleles (Rast 1988; Genda et al. 2007; Antignus et al. 2008). Plants homozygous for allele L+ are susceptible to all described pathotypes (P0, P1,2, P1,2,3, and P1,2,3,4) (i.e., they are rr under the GFG model), plants with a L1/- genotype are resistant only to pathotype P0, L2/- plants are resistant only to pathotypes P0 and P1 and so on. Pathotype P1,2,3,4 is able to infect pepper genotypes with each of the four resistance alleles. L-gene resistance is dominant or semidominant and is expressed as a hypersensitive response, which is elicited, for all L alleles, by the virus coat protein (CP) (Berzal-Herranz et al. 1995; Cruz et al. 1997; Gilardi et al. 2004; Matsumoto et al. 2008).

Our group has reported conclusive evidence for resistance-breaking costs in pepper-infecting tobamoviruses (Fraile et al. 2011). This evidence was derived from comparison of changes in pathotype frequency and in the fraction of the crop carrying the various resistance alleles, over more than 20 years in South-East (SE) Spain. Also, costs in the within-host multiplication component of viral fitness were estimated in single infection and in competition, for isolates representative of the three pathotypes present in the virus population: P0, P1,2, and P1,2,3. In the susceptible host genotype L+ L+, isolates of pathotype P1,2,3 were outcompeted by isolates of pathotype P1,2, and both were outcompeted by isolates of pathotype P0 (Fraile et al. 2011).

Here, two interrelated hypotheses were analyzed, using the set of field-collected isolates described by Fraile et al. (2011): 1) as the CP is the virus AVR factor for L-gene resistance, CP changes associated with resistance-breaking would affect the stability of virus particles; 2) particle stability would be positively correlated with virus survival in the soil. Results support both hypotheses and provide evidence of differential survival in the environment of the various virus pathotypes, which would affect the evolution of resistance-breaking and, hence, of host range expansion.

**Results**

**Model Predictions and Experimental Evaluation of the Stability of Tobamovirus Particles of Isolates from Pathotypes P0, P1,2, and P1,2,3**

In the population of pepper-infecting tobamoviruses in SE Spain, all P0 isolates belonged to Tobacco mild green mosaic virus (TMGMV) while all isolates of pathotypes P1,2 and P1,2,3 belonged to Pepper mild mottle virus (PMMoV) (Fraile et al. 2011). All P1,2,3 isolates differed from all P1,2 isolates by the mutation M138N in the CP, previously reported to be sufficient to acquire the ability to break the resistance conferred by allele L3 (Berzal-Herranz et al. 1995). The nucleotide sequence of the CP gene was determined for 21 P0 isolates, 32 P1,2 isolates, and 12 P1,2,3 isolates, according to the relative frequency of these pathotypes in the virus population (Fraile et al. 2011). Seven sequence variants or haplotypes were detected for TMGMV P0 isolates, the two most frequent ones comprising 42.8% and 28.6%, respectively, of the P0 population. In the case of PMMoV, five CP haplotypes were identified for P1,2 isolates and two for P1,2,3 isolates, the most
frequent haplotype accounting for 87.5% and 91.7% of the P1,2 and P1,2,3 isolates, respectively (table 1).

Analysis of Molecular Interactions among CP Subunits in Virus Particles

The particle structure of isolate U2 of TMGMV (U2-TMGMV), which has been determined at 3.5 Å resolution by X-ray crystallography (Pattanayek and Stubbs 1992), was used as reference for analyses of interactions between subunits in the virus particle for the various CP sequence variants. Over the seven TMGMV P0 haplotypes, amino acid differences with U2-TMGMV were found at ten positions of the 158 amino acids CP of U2; four differences were common to all seven haplotypes, and all of them involved nonconservative amino acid changes (supplementary table S1, Supplementary Material online, and fig. 1A). Over the seven PMMoV P1,2 and P1,2,3 haplotypes, amino acid differences relative to U2-TMGMV CP were found in 33 positions, 26 of which were common to all haplotypes, and 14 resulted in nonconservative amino acid changes (supplementary table S1, Supplementary Material online, and fig. 1B). Despite these differences, the modeled geometries for either TMGMV or PMMoV haplotypes superimposed indistinguishably on that of U2-TMGMV (root mean square deviation computed for backbone atoms ~0.15 Å), revealing that these sequence differences affected only the conformation of side chain rotamers.

Next, the interactions established among each of the amino acids in a focal CP subunit (subunit A) with four other neighbor subunits forming a core pentamer (subunits D, L, R, and U) (fig. 1C) were calculated for each CP haplotype. Interactions were divided into axial contacts, that is, those established with subunits in different turns of the helical structure (contacts between subunits A-D, A-U, L-U, and D-R in fig. 1C), and side contacts, that is, those established with subunits within the same helix turn (contacts between subunits A-L and A-R in fig. 1C). The total possible axial and side interactions are presented in supplementary tables S2 and S3, Supplementary Material online, and are summarized in table 1 and figure 2. The computation of these results indicated that for all seven TMGMV CP haplotypes, axial interactions were dramatically weakened as compared with U2-TMGMV, because 15 (7 hydrogen bonds [HB] and 8 Van der Waals [vdW] contacts) of the 37 axial interactions that were established between U2-TMGMV subunits were lost. On the other hand, side contacts were strengthened by new strong HB. Minor differences in the number of HB and vDW contacts in side interactions existed between haplotypes, interactions being slightly stronger for the most frequent haplotype (haplotype 1) and weakest for the second most frequent haplotype (haplotype 4) compared with the rest. For the seven PMMoV haplotypes, axial interactions were significantly stronger than in U2-TMGMV, increasing from 37 to 48–52 contacts, with a net gain of 5–6 HB. Side interactions were also considerably increased respective to U2-TMGMV, from 57 to 80–82 contacts, with a net gain of 6–7 HB. It must also be noted that although the number of ion pairs was strictly conserved (table 1), their associated distance in four out of the six ion pairs was significantly shorter in all P0, P1,2, and P1,2,3 haplotypes than in U2-TMGMV, while the remaining two ion pairs displayed similar distances (supplementary tables S2 and S3, Supplementary Material online). This result indicates an additional strengthening of the interactions among subunits in all the PMMoV CP haplotypes as compared with U2-TMGMV. Although all the PMMoV CP haplotypes will

Table 1. Summary of Axial and Side Interactions among CP Subunits in Core Pentamers in Virus Particles of Pepper-Infecting Tobamoviruses of P0 Pathotype (TMGMV isolates, numbers 1–7) and of P1,2 and P1,2,3 Pathotypes (PMMoV isolates, numbers 8–14).

<table>
<thead>
<tr>
<th>Haplotype and Pathotype</th>
<th>Reference Isolate</th>
<th>Frequencya</th>
<th>Axial</th>
<th>Side</th>
<th>Total Contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ion Pairs Hydrogen Bonds Van der Waals Contacts</td>
<td>Ion Pairs Hydrogen Bonds Van der Waals Contacts</td>
<td></td>
</tr>
<tr>
<td>1 TMGMV P0</td>
<td>P84/9</td>
<td>0.428</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>2 TMGMV P0</td>
<td>P96/49</td>
<td>0.047</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>3 TMGMV P0</td>
<td>P98/12</td>
<td>0.047</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>4 TMGMV P0</td>
<td>P98/11</td>
<td>0.286</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>5 TMGMV P0</td>
<td>P01/13</td>
<td>0.095</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>6 TMGMV P0</td>
<td>P04/17</td>
<td>0.047</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>7 TMGMV P0</td>
<td>P96/53</td>
<td>0.047</td>
<td>2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>8 PMMoV P1,2</td>
<td>P90/4</td>
<td>0.875</td>
<td>2</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>9 PMMoV P1,2</td>
<td>P85/29</td>
<td>0.031</td>
<td>2</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>10 PMMoV P1,2</td>
<td>P87/28</td>
<td>0.031</td>
<td>2</td>
<td>22</td>
<td>27</td>
</tr>
<tr>
<td>11 PMMoV P1,2</td>
<td>P87/18</td>
<td>0.031</td>
<td>2</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>12 PMMoV P1,2</td>
<td>P87/15.2</td>
<td>0.031</td>
<td>2</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>13 PMMoV P1,2,3</td>
<td>P89/4</td>
<td>0.083</td>
<td>2</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>14 PMMoV P1,2,3</td>
<td>P01/11</td>
<td>0.917</td>
<td>2</td>
<td>22</td>
<td>26</td>
</tr>
<tr>
<td>U2-TMGMV (Ref.)</td>
<td>NC_001556</td>
<td></td>
<td>2</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>

a Frequencies are calculated over the population of each of the three pathotypes. Accession number for the nucleotide sequence of coat protein genes of all isolates are FR671380-FR671402, FNS94854-FNS94889, and HG1449-HG14456.
form markedly more stable discs and significantly more stable global assemblies than U2-TMGMV, differences in stability among haplotypes did not allow their ranking similarly according to the strength of axial and side contacts (table 1). The most frequent P1,2,3 haplotype, haplotype 14, was predicted to establish both axial and side interactions somewhat weaker than the most frequent P1,2 haplotype (haplotype 8).

In summary, comparisons of protein–protein interactions in CP sequence variants of isolates of pathotypes P0, P1,2, and P1,2,3 with those of isolate U2-TMGMV predicted that the structure of particles of P0 isolates will be much less stable than the structure of particles of P1,2 and P1,2,3 isolates due to overall weaker HB and VdW contacts, both axial and side. In addition, the structure of particles of the major P1,2,3 haplotype will be slightly less stable than that of most P1,2 isolates due to fewer axial and side interactions.

**Experimental Evaluation of the In Vitro Stability of Virus Particles**

The above predictions of particle stability were tested experimentally. It has been long known that particles of tobamoviruses disassemble in conditions of basic pH or high ionic strength, as analyzed in detail for Tobacco mosaic virus (TMV) (Perham and Wilson 1976; Ohno and Okada 1977; Blowers and Wilson 1982). The kinetics of particle disassembly were analyzed by incubating particles of representative isolates of

---

**Fig. 1.** (A and B) Ribbon diagram of the structure of the CP subunit of pepper-infecting tobamoviruses modeled upon the crystal structure of U2-TMGMV. Sequence differences between U2-TMGMV and P0 pathotype (TMGMV isolates, A) and between U2-TMGMV and P1,2 and P1,2,3 pathotypes (PMMoV isolates, B) are indicated depicting changed residues as sticks with the following color code. Cyan: major changes in all the sequences, blue: major changes in some sequences, pink: conservative changes in all the sequences, magenta: conservative changes in some sequences. (C). Molecular surface of a three-turn section of the virus particle of U2-TMGMV and pepper-infecting tobamoviruses indicating five CP subunits that integrate a core pentamer used to analyze protein–protein interactions. Left: side view, right: top view.
pathotypes $P_0$, $P_{1,2}$, and $P_{1,2,3}$ in both Tris–HCl pH 8.75 and in 6 M urea for different times and monitoring the degree of particle disassembly by agarose gel electrophoresis (see Materials and Methods). To represent the virus population, isolates were randomly chosen among those with a known pathotype and CP haplotype. For the more diverse TMGMV $P_0$ population, seven isolates were assayed, of which five belonged to the two most frequent CP haplotypes (three of the first and two of the second). For the PMMoV $P_{1,2}$ and $P_{1,2,3}$ population, five and four isolates were assayed, respectively, with the most frequent CP haplotypes of pathotypes $P_{1,2}$ and $P_{1,2,3}$ being represented by four isolates each (supplementary table S4, Supplementary Material online).

Results did not differ for the various isolates within each pathotype, but strongly differed among pathotypes as shown in figure 3. The particles of TMGMV $P_0$ isolates were the less stable, with disassembly in both pH 8.75 and 6 M urea being apparent after only 10 and 5 min incubation, respectively, with disassembly being complete at 60 min. On the contrary, the particles of PMMoV $P_{1,2}$ isolates only began to destabilize in 6 M urea after 60 min incubation and at pH 8.75 after 2 h. The particles of PMMoV $P_{1,2,3}$ isolates were also more stable than those of $P_0$ isolates but showed a diminished stability as compared with those of PMMoV $P_{1,2}$ isolates: disassembly in basic pH conditions started after 30 min and in 6 M urea started after 45 min of incubation.

Hence, these results are consistent with the predictions of model analyses, except that the kinetics of disassembly in the analyzed conditions did not reveal the minor differences in

---

**Fig. 2.** Ribbon diagram of the model structure of the CP subunit of pepper-infecting tobamoviruses of $P_0$ pathotype (TMGMV isolates, A) and $P_{1,2}$ and $P_{1,2,3}$ pathotypes (PMMoV isolates, B). Residues with two or more contact differences in protein–protein interactions between the pathotypes and U2-TMGMV are depicted as sticks with the following color code. Cyan: residues with more contacts in pathotypes than in U2-TMGMV, red: residues with fewer contacts in pathotypes than in U2-TMGMV.

---

**Fig. 3.** Kinetics of virus particles disassembly in Tris–HCl pH 8.75 or 6 M urea for representative isolates of pepper-infecting tobamoviruses of $P_0$ pathotype (TMGMV isolates) and of $P_{1,2}$ and $P_{1,2,3}$ pathotypes (PMMoV isolates). Data shown are for isolates P84/9 ($P_0$ TMGMV isolate, CP haplotype 1), P90/4 ($P_{1,2}$ PMMoV isolate, CP haplotype 8), and P02/4 ($P_{1,2,3}$ PMMoV isolate, CP haplotype 14).

---

**Analysis of Virus Accumulation in Different Plant Organs, and of Particle Survival in the Soil, of Isolates from Pathotypes $P_0$, $P_{1,2}$, and $P_{1,2,3}$**

To test whether the differences in particle stability, predicted by structural analyses and experimentally determined in vitro, were related to the long-term survival of virus particles in the soil, five isolates of each of the pathotypes $P_0$, $P_{1,2}$, and $P_{1,2,3}$ randomly chosen from the available collection were
inoculated in susceptible L+ L+ plants. The experiment included the same isolates analyzed for particle stability in vitro, except that P0 isolates P92/10 of haplotype 1 and P04/17 of haplotype 6 were not included, and P1,2,3 isolate P96/41, of haplotype 14, was added. Fifteen days post-inoculation (dpi) virus accumulation was quantified in systemically infected leaves and in the roots to analyze resistance-breaking costs in the within-host multiplication component of viral fitness. In addition, after removing the plants, the soil in which they had grown was kept for 6 months in the greenhouse, both in wet and in dry conditions. After this period, virus particles were extracted from the soil, and their viability was quantified by local lesion assay in Nicotiana tabacum cv. Xanthi-nc.

Data of virus accumulation in systemically infected leaves reproduced those previously published based on a different set of isolates (Fraile et al. 2011): in single infection, the accumulation of isolates of pathotype P0 was significantly higher than the accumulation of isolates of either pathotype P1,2 or P1,2,3 (F2,21 = 7.33, P = 1 × 10⁻³), which did not differ significantly (P = 0.973) (fig. 4 and supplementary table S5, Supplementary Material online). The same trend was found for virus accumulation in the roots, which was significantly higher for P0 isolates than for P1,2 or P1,2,3 isolates (F2,75 = 28.55, P < 1 × 10⁻⁵), with no differences between the last two pathotypes (P = 0.817) (fig. 4 and supplementary table S5, Supplementary Material online). Interestingly, virus multiplication in the roots was significantly less efficient than in the leaves (F1,146 = 16.85, P < 1 × 10⁻⁴), accumulation at 15 dpi being between one-half and one-tenth that in the leaves, depending on the pathotype (F1,50 = 3.45, P = 0.070; F1,48 = 7.24, P = 0.010; F1,48 = 68.99, P < 1 × 10⁻⁴ for P0, P1,2, and P1,2,3 isolates, respectively).

Survival in the soil was assessed by a necrotic local lesion (nll) infectivity assay in Xanthi-nc tobacco leaves. The number of nll induced by virus particles that survived for 6 months either in wet or dry soil depended on the pathotype (F2,447 = 14.58; P < 1 × 10⁻³), but not on the state at which the soil was maintained (wet or dry) (F1,447 = 0.00, P = 0.983) nor on the interaction between pathotype and state of the soil (F2,447 = 0.01, P = 0.986) (fig. 4 and supplementary table S5, Supplementary Material online). The number of infectious particles after 6 months, as estimated by nll assay, was significantly higher for P0 isolates than for P1,2 or P1,2,3 isolates (P < 2 × 10⁻⁴), and significantly higher for P1,2 than for P1,2,3 isolates (P = 0.023). This relationship was not the same as for within leaf or within root accumulation. The relative number of infectious particles that survived in the soil was compared with the value expected from the relative accumulation in the roots (see Materials and Methods). It was found to be significantly lower than expected for P0 as compared with P1,2 (χ² = 21.00, P = 2 × 10⁻⁴ in a Fisher exact test) and marginally significantly higher for P1,2 than for P1,2,3 (χ² = 3.10, P = 0.107). Hence, relative survival in the soil is more efficient for P1,2 isolates than for P1,2,3 and for P1,2,3 than for P0 isolates.

**Comparison of the Infectivity of Isolates from Pathotypes P0 and P1,2**

It has been reported that TMV mutants with hyperstable particles had a reduced infectivity (Culver 2002). Hence, it could be that the much higher stability of particles of PMMoV P1,2 and P1,2,3 isolates relative to TMGMV P0 isolates resulted in a diminished infectivity, that is, in a trade-off between survival and infectivity. To test this hypothesis, the infectivity of four of the five P0 and five P1,2 isolates in the experiment above was compared by nll assay. Results (fig. 5) indicated that the concentration–infectivity curves were not significantly different for either set of isolates (F1,70 > 0.45, P = 0.507). Hence, differences in particle stability that translate into differences in particle survival do not affect virus infectivity.

![Fig. 4. Within-host multiplication and survival in the soil of pepper-infecting tobamoviruses of P0 pathotype (TMGMV isolates) and of P1,2 and P1,2,3 pathotypes (PMMoV isolates).](image)

![Fig. 5. Infectivity of pepper-infecting tobamoviruses of P0 pathotype (TMGMV isolates) and of P1,2 pathotypes (PMMoV isolates). Infectivity was estimated by the number of necrotic local lesions caused in Nicotiana tabacum Xanthi-nc by four isolates of each pathotype at different inoculum concentrations. Infectivity–dilution curves are represented.](image)
Discussion

There is abundant evidence for across-host fitness trade-offs in plant viruses. Evidence derives mostly from serial passage experiments in different hosts, from the analysis of engineered mutants with altered host ranges, and, less frequently, from the analysis of field isolates which differ in host range. Most analyses focus on the capacity of the virus to infect previously resistant host plant genotypes, that is, from the analysis of resistance-breaking genotypes (García-Arenal and Fraile 2010). Resistance-breaking is obviously associated with a fitness increase in the resistant host genotype but may have costs in the nonresistant host genotypes. When quantified, resistance-breaking costs in plant viruses have been found to be sufficiently high that they might effectively limit the evolution of new host ranges (Jennner et al. 2002; Ayme et al. 2006; Janzac et al. 2010; Poulicard et al. 2010, 2012; Fraile et al. 2011). Across-host fitness trade-offs in plant viruses are best explained by antagonistic pleiotropy (Whitlock 1996), that is, by opposite phenotypic effects of mutations in different environments. Evidence indicates that antagonistic pleiotropy is the most important mechanism generating across-host trade-offs in RNA and small DNA viruses. This is likely an unavoidable consequence of the nature of viral genomes, which are small, with highly compact genetic information, yet encoding only a few multifunctional proteins that need to interact with host components to fulfill their function (Sacristán and García-Arenal 2008; Elena et al. 2009; Fraile and García-Arenal 2010). To our knowledge, all reported analyses of across-host trade-offs in plant viruses have focused on the within-host multiplication component of the viral fitness. However, trade-offs are expected to occur between different fitness components of parasites (Alizon et al. 2008; Kochin et al. 2010), and these trade-offs may be favored by the multifunctionality of viral proteins. Thus, current emphasis on within-host multiplication to the exclusion of other fitness components might be a serious limitation in our understanding of host range evolution, including resistance-breaking, in viruses. Survival is, together with reproduction, a key component of fitness (Crow 1985). However, survival of free-living stages of parasites has received attention only recently as a factor in parasite evolution (Walther and Ewald 2004; de Paepe and Taddei 2006; de Paepe and Taddei 2006; Montarry et al. 2007) and, to our knowledge, has not been analyzed in plant viruses.

This work analyzed whether host range expansion of pepper-infecting tobamoviruses on Capsicum genotypes with different L-gene resistance alleles is associated with differential survival of virus particles in the soil. The rationale for this hypothesis is that the capacity of tobamoviruses to infect and multiply, that is, break the resistance, in pepper genotypes with different L-gene resistance alleles, depends on the ability of virus particles to infect and multiply in different hosts, and, therefore, on the within-host multiplication component of the viral fitness.

Epidemic in the next crop. Previous work from our group on the population dynamics of viral pathotypes in the pepper-infecting tobamovirus population in SE Spain over 20 years indicated a rapid evolution of the virus in response to the deployment of different resistance alleles. The comparison of pathotype frequency with the proportion of cultivars carrying different resistance alleles strongly suggested that resistance-breaking was associated with fitness penalties. Also, quantifying the multiplication in nonresistant plant genotypes of virus isolates representing the three pathotypes present in SE Spain, P0, P1,2, and P1,2,3, provided conclusive evidence of within-host multiplication costs of resistance-breaking (Fraile et al. 2011).

This work shows that survival in the soil differs between three tobamovirus pathotypes and correlates with the in vitro stability of virus particles. For both traits, the analyzed pathotypes ranked P1,2 > P1,2,3 > P0. This result agrees with the hypothesis that virus survival in the environment correlates positively with particle stability (de Paepe and Taddei 2006) and with the observation that all plant viruses that survive in the soil have highly stable particles (Hull 2002, p. 546). Importantly, in vitro stability of virus particles was according to predictions from structure modeling of P0, P1,2, and P1,2,3, which are the structure determined for U2-TMGMV (Pattanayek and Stubbs 1992). For TMV it has been shown that particle disassembly both in Tris–HCl pH 8.75 and in 6 M urea proceeds in an orderly manner, primarily from the end of the rod corresponding to the 5′ end of the virus RNA and, only later and more slowly, from the 3′ end. Disassembly proceeded in discrete steps, with stable intermediates occurring due to regions of more stable contacts between CP subunits and the virus RNA, and there was a fraction of particles that did not disassemble under any condition (Perham and Wilson 1976; Ohno and Okada 1977; Blowers and Wilson 1982). This pattern suggests that disassembly is due primarily to the destabilization of axial interactions among CP subunits. Our experimental results and model predictions are consistent with this hypothesis: many fewer axial HB and VdW contacts were established in TMGMV than in PMMoV particles, and PMMoV particles were more stable at high pH and high molar urea than TMGMV ones. Also, the lower in vitro stability of PPMoV haplotype 14 relative to haplotype 8 correlates with axial interactions in haplotype 8 being predicted to be reinforced by 1 HB and 2 VdW contacts relative to haplotype 14. On the other hand, weaker predicted side interactions in haplotypes 4 and 6 of TMGMV as compared with haplotype 1 or in haplotype 10 of PPMoV as compared with haplotype 8, or stronger side contacts predicted for haplotype 14 relative to haplotype 8, were not detected by the kinetics of their in vitro disassembly (data not shown and fig. 3). Taken together, these data indicate that axial interactions between CP subunits in virus particles are the primary determinant of their in vitro stability. Note that the in vitro stability of virus particles will not be the only determinant of survival in the environment; other factors, for instance differential particle disassembly during plant tissue degradation in the soil, or differential interaction of particles with soil components (Yoshimoto et al. 2012) could be envisioned...
to affect survival. However, a first conclusion of our results is that the strength of axial interactions between CP subunits in tobamovirus particles is a good predictor of virus survival in the environment.

Another important result of this work is that survival and within-host multiplication in nonresistant hosts rank differently for the three tobamovirus pathotypes: P_{1,2} > P_{1,2,3} > P_0 for survival and P_0 > P_{1,2} > P_{1,2,3} for within-host accumulation, as estimated from competition experiments (Fraile et al. 2011). Trade-offs between multiplication and survival have been reported for viruses infecting bacteria and animals (de Paepe and Taddei 2006; Brown et al. 2009; Heineman and Brown 2012; Handel et al. 2013; Ogbunugafor et al. 2013) on the basis of negative correlations between these traits. The present results show a more complex relationship between multiplication and survival and between both components of viral fitness and host range, depending on the specific virus genotypes. Hence, for P_{1,2} isolates, within-host multiplication costs of resistance-breaking will be traded against increased survival, while in the case of P_{1,2,3} isolates, both within-host multiplication and survival will combine to increase the costs of resistance-breaking. Similarly, the advantage of P_0 isolates in within-host multiplication rates in nonresistant hosts will be countered by low survival. It is relevant that no trade-off between survival and infectivity was detected, which would have added a further level of complexity to interactions among different fitness components. Importantly, and at odds with previous reports of survival-multiplication trade-offs in other viruses, our results allow dissection of the mechanisms underlying the relationships among fitness components. Results indicate pleiotropic effects of amino acid substitutions in two different functions of the virus CP: resistance-breaking, that is, the capacity to avoid recognition by proteins encoded by L-gene resistance alleles, and survival. Pleiotropy is clearly antagonistic in P_{1,2,3}: the mutation M138N in the CP is sufficient to acquire the ability to break L^* allele resistance (Berzal-Herranz et al. 1995) and to destabilize axial contacts between CP subunits (supplementary table S2, Supplementary Material online), decreasing particle stability and survival. However, the sense of the pleiotropy for the different pathotypes is not always antagonistic but differs in different environments: across hosts with different resistance alleles and within hosts versus the environment. The consequence with respect to viral fitness is a complex one. Fitness penalties of traits that provide an environment-dependent benefit have been shown to play important roles in the interactions between different organisms or genotypes. For instance, environment-dependent fitness costs associated with the production of, and with resistance to, bacteriocins in bacteria or killer toxins in yeasts determine the competition among producer, susceptible, and resistant genotypes. Depending on conditions, for example, population structure-connectivity or genotype frequency, such costs may result in complex nontransitive interactions with cyclic dominance according to a rock-paper-scissors game (Kerr et al. 2002; Kirkup and Riley 2004; Brown et al. 2009; Nahum et al. 2011).

Differential survival will alter the relative amounts of isolates of the three tobamovirus pathotypes in the soil, to the relative accumulation in planta. The quantitative relevance of differences in survival will depend on at least four factors: 1) the input of virus particles into the soil, which will derive primarily from the roots of infected plants, as in most cropping systems stems and leaves are removed after harvest, 2) the genetic composition of the host populations, which will modulate virus input into the soil, 3) the length of the period between subsequent crops; and 4) the relative importance of primary infections from soil inoculum and secondary infections by contact transmission between plants, which will be affected by the efficiency of rouging. Survival in the environment is critical for the transmission of many human and animal pathogens, including viruses (Walther and Ewald 2004). However, its role on infection dynamics and pathogen evolution has been overlooked, and only recently differences in survival among virus genotypes are being considered in epidemiological and evolutionary models of viruses such as avian influenza or baculoviruses, whose transmission dynamics rely on survival in the environment (Breban et al. 2009; Fuller et al. 2012; Elderd 2013; Handel et al. 2013). A recognized limitation for the development of such models is a paucity of empirical data on survival of different viral genotypes (Fuller et al. 2012), such as those reported here. Survival in the soil as infectious particles is a key step in the epidemiology of economically relevant plant viruses in genera such as *Tobamovirus*, *Potexivirus*, or *Tombusivirus* (Kurstak 1981). Extra-host survival might also have a role in the dynamics and evolution of viruses with other mechanisms of transmission. For example, a relationship between particle stability and transmissibility has been reported for *Cucumber mosaic virus* (Ng et al. 2000; Bricault and Perry 2013), a nonpersistently transmitted virus, which suggests that it must survive as infectious particles while retained in the cuticle of the aphid vector mouth parts. Hence, parameters related to survival should be quantified for different virus genotypes and need to be considered for a good understanding of their population dynamics and evolution.

A major conclusion of this work is that changes in resistance in the host population exert a selection pressure on the virus population resulting in host range expansion through resistance-breaking, as previously reported (Fraile et al. 2011), but also select for viral traits, such as survival in the environment, which are unrelated to the host–virus interaction. How general this novel finding will be remains to be established, but it certainly should be considered in future analyses of host–parasite coevolution. Because selection on traits related and unrelated to the host–virus interaction may be in the same or opposite directions, the result may be increased or diminished across-host trade-offs. Hence, the interaction between fitness components related and unrelated to virus–host interactions may favor or hinder host range expansion, introducing a new uncertainty in the prediction of virus evolution and of virus emergence.
Material and Methods

Model Analysis of Interactions among CP Subunits in Virus Particles

The structure of the CP subunit assembled into virus particles was modeled for the 14 CP sequence variants by homology modeling with SwissModel (Arnold et al. 2006; Kiefer et al. 2009) using the crystal structure of U2-TMGMV, PDB code 1VTM (Pattanayek and Stubbs 1992) as a template. The biological assembly with helical symmetry made of 49 subunits in three turns of the helix was generated for all the P0, P1,2, and P1,2,3 pathotype CP haplotypes using the 49 transformation matrices in the 1VTM entry. A core pentamer of CP subunits from the biological assembly was then selected to compute protein–protein interactions taking one central subunit (labeled A in fig. 1C) together with its up, down, left, and right neighbor subunits (labeled U, D, L, and R, respectively, in fig. 1C). Protein–protein contacts were then calculated on the 14 core pentamers with the PDB2PQR program (Dolinsky et al. 2004) and separated into two groups for the analysis: axial contacts, that is, those established with subunits which are in different turns of the helical structure (contacts A-U, A-D, L-U, and D-R), and side contacts, that is, those established with subunits within the same helix turn (contacts A-L and A-R) (fig. 1C). The criteria used by PDB2PQR to consider the existence of a contact were the following: 1) for HB, cut-offs of 30° for acceptor–hydrogen–donor angles and 3.4 Å for donor–acceptor distance, H-bonds being considered weak, moderate, or strong if that distance was >3.0, between 3.0 and 2.5, and ≤2.5 Å, respectively, 2) for ion pairs, the cut-off is cation–anion distance 4.0, and 3) for VdW contacts, an interatomic distance ≤3.5 Å (Dolinsky et al. 2004).

Analysis of the In Vitro Stability of Virus Particles

Virus particles were purified as in Bruening et al. (1976), and their in vitro stability was analyzed for seven P0, five P1,2, and four P1,2,3 isolates (supplementary table S4, Supplementary Material online). These isolates were multiplied in Nicotiana clevelandii plants and particles were purified simultaneously for all of them to avoid differences in stability attributable to different storage conditions and times. Particle stability was analyzed by incubating 200 μg of particles in two different conditions for 6 months, and after this period, virus parts that were then maintained in two different states, either dry, with no further watering from the day of plant harvest, or wet, with watering to field capacity twice a week. The different soils were kept in the greenhouse at the conditions specified earlier for 6 months, and after this period, virus

Quantification of Virus Accumulation in Pepper Leaves and Roots

The multiplication of the various tobamovirus isolates was analyzed in pepper cv Doux des Landes, which is homozygous for the susceptibility allele L+. Plants were inoculated at the two first true leaves with 0.2 μg of freshly purified virus particles in 0.1 M phosphate buffer pH 7.2 and were maintained in a greenhouse (20–25°C, 16 h light). The experiment involved five isolates of each of pathotypes P0, P1,2, and P1,2,3 plus mock inoculated controls, with five replicated plants per treatment. Treatments were distributed randomly in the greenhouse using a random number table. At 15 dpi plants were harvested, with roots being carefully separated from the soil. Virus accumulation was quantified as viral RNA accumulation via quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), in both systemically infected leaves and in roots, from which total nucleic acid extracts were obtained using Trizol reagent (Life technologies, Carlsbad, CA, USA) according to manufacturer’s protocol. For each sample, 0.5–3 ng of total RNA were utilized with Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s recommendations in a final volume of 10 μl. Assays were performed in triplicate on a LightCycler 480 II real-time PCR system (Roche, Indianapolis, IN, USA). Relative levels of viral RNA were deduced from standard curves produced using a set of serial dilutions of plant material (leaf or root) as well as for each viral pathotype using equal amount mixes of purified viral RNA of the five isolates of each pathotype. Primers b-tubF C.a. (GAGGGTGAGTGAGCAGTTC) and b-tubR C.a. (CTTCATCGTCATCTGCTGTC) were used to amplify 166 nt of the Capsicum annuum b-tubulin (GenBank: EF495259) transcript that was then used as internal control. In order to quantify virus accumulation in the various samples, primers P0bF (GCACCGAATACTACTGAAATCG), P0bR (ATTGGCCAAATTATTTAGGAAA), P12/123F (AACCTGCCGAGACGCTT), P12/123R (GAGTTGTAGCCAGGCTT), P12/123f2R (GAGTTGTAGCCAGGCTT), P12/123fF (AACTGCCAGACGCTT), and P12/123dF (TGACACGCTT), P12/123dR (TCGAAAATCCTACTGCCT), P12/123f2R (GAGTTGTAGCCAGGCTT), P12/123fF (AACTGCCAGACGCTT), and P12/123dF (TGACACGCTT), P12/123dR (TCGAAAATCCTACTGCCT) were designed to amplify 89 nt, 157 nt, and 138 nt of the P0-CP (GenBank: FNS94861), P1,2-CP (GenBank: FNS94868), and P1,2,3-CP (GenBank: FR671386) pathotypes, respectively. Reactions including no template and no reverse transcriptase were included in each trial. Thermal parameter for RT-PCR amplification were 50°C for 10 min, 95°C for 3 min, and 40 cycles of 95°C for 5 s and 60°C for 10 s. Dissociation curves were generated to ascertain that only a single product was produced in each case.

Quantification of Virus Survival in the Soil

After harvesting of the plants for the above-mentioned experiment, soil from five replicates was divided in two equal parts that were then maintained in two different states, either dry, with no further watering from the day of plant harvest, or wet, with watering to field capacity twice a week. The different soils were kept in the greenhouse at the conditions specified earlier for 6 months, and after this period, virus
particles were extracted following a procedure modified from Allen (1981): 20 ml of previously homogenized soil was stirred for 20 min in either 40 ml (dry soil) or 30 ml (wet soil) of 1 M phosphate buffer pH 7.0 at room temperature. After filtering, the liquid phase was centrifuged at 11,300 × g for 10 min, and the supernatant was further centrifuged at 145,500 × g for 1.5 h. The resulting pellet was resuspended in 1.5 ml of 1 mM ethylenediaminetetraacetic acid (EDTA).

Virus survival was quantified from these preparations by nll assay in plants of N. tabacum cv. Xanthi-nc, which carry the resistance gene N. Two consecutive fully expanded leaves per plant were inoculated, with one treatment per half leaf. Half leaves were inoculated with 20 μl of the soil virus preparation. Three repetitions were done for each of the 150 virus preparations, in a fully randomized design. Four dpi, when nll were apparent, leaves were harvested and nll were counted.

Quantification of Infectivity

Four P₀ and four P₁,2 isolates randomly chosen between those used in the previous assay, belonging to the most frequent haplotype in each pathotype, were multiplied in N. clevelandii plants, and particles were purified simultaneously for all of them to avoid differences attributable to different storage times. Initial viral concentration of 90 μg/ml resuspended in 1 mM EDTA was then diluted at 1/10, 1/30, 1/90, 1/270, 1/810, and 1/1240. Twenty microliters of each dilution were inoculated in half leaves of two consecutive fully expanded leaves per plant of N. tabacum cv. Xanthi-nc. Ten replicated inoculations were done per treatment in a fully randomized design. Four dpi, when nll were apparent, leaves were harvested and nll were counted.

Statistical Analyses

Data on virus accumulation and on number of necrotic local lesions, as well as their transformations, were normally distributed and showed homogeneity of variances among pathotypes. Therefore, differences in viral accumulation according to pathotype, type of tissue (leaf or root), or soil condition (dry or wet) were analyzed using General Linear Models (GLM). Differences in viral accumulation according to tobamovirus pathotype (P₀, P₁,2, and P₁,2,3), type of tissue, or soil conditions and the interactions between these factors were analyzed by a GLM in a full factorial model, considering all factors as fixed effects. In addition, similar analyses were carried out using a nested model considering isolate as nested to pathotype. Because GLM comparisons of these data led to similar results and conclusions as did nested models, for simplicity, only GLM analyses are shown. To determine whether values of analyzed traits were significantly different among classes within each factor, Least Significant Difference (LSD) analyses were employed in all cases (Sokal and Rohlf 1995). Relationship between infectivity and virus concentrations was tested by bivariate analysis considering linear and nonlinear models. Infectivity curves were compared using analysis of variance to test the equality of slopes and intercepts. To do so, exponential curves were transformed into linear regressions by calculating the decimal logarithm of the number of necrotic local lesions. A χ² test was used to compare the relative number of infectious particles that survived in the soil with the value expected from the relative accumulation in the roots between pairs of pathotypes. The expected value was obtained by summing the observed number of infectious particles A and B in the soil for each pair of pathotypes and dividing the resulting number according to the ratio of the accumulation in the roots of these two pathotypes. All statistical analyses were performed using the statistical software package SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Supplementary Material

Supplementary tables S1–S5 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

Beatriz Simón, Miguel Ángel Mora, and Antolín López-Quiros provided excellent technical assistance. The authors thank Prof. Mark Wilkinson, CBGP, for critical reading of a former version of the manuscript. This work was in part funded by grant AGL2008-02458 (Plan Nacional de I + D + I, Spain) to F.G.-A. J.-M.H. was supported by a Marie Curie-COFUND contract from the European Commission-Universidad Politécnica de Madrid (FP7). I.P. was supported by the Ramon y Cajal Programme from the Spanish Government.

References


