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SHORT REVIEW

Experimental evolution of plant RNA viruses

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Undoubtedly, viruses represent a major threat faced by human and veterinary medicines and by agronomy. The rapid evolution of viruses enables them to escape from natural immunities and from state-of-the-art antiviral treatments, with new viruses periodically emerging with deadly consequences. Viruses have also become powerful and are increasingly used tools in the field of experimental evolution. A growing body of evidence points that the evolution of viruses is mainly determined by key features such as their compacted genomes, enormous population sizes, and short generation times. In addition, RNA viruses also present large selection coefficients, antagonistic epistasis, and high mutation rates. Most of this knowledge comes from studies that have used either bacteriophages or animal viruses in cell cultures as experimental systems. However, plant viruses provide almost identical advantages for evolutionary studies and, in addition, offer an invaluable tool for studying the interplay between viruses and pluricellular hosts. Without seeking to be exhaustive, here we summarize some peculiarities of plant viruses and review recent experiments that have explored important questions on evolution, such as the role of deleterious mutation and neutrality, the effect of different transmission modes in the evolution of virulence, and the heterogeneous selective constraints imposed by multiple hosts.
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Introduction

RNA viruses are the most abundant parasites infecting humans, farm animals, and cultivated plants (Domingo and Holland, 1997). Despite tremendous economical efforts, the number of eradicated viruses is quite limited. Furthermore, the perspectives for future eradications are counterbalanced by the emergence of new viruses (Murphy and Nathanson, 1994). The fact that few viruses can be effectively controlled and the pervasive emergence of new viruses are likely consequences of their remarkable ability to evolve. Immune-escaping and antiviral-resistant strains arise soon after viral populations are challenged (Althaus and Bonhoeffer, 2005) and viruses may jump the species barrier from their natural reservoir hosts to new ones (Daszak *et al*., 2000). Besides seeking information on these questions, the study of RNA virus evolution may help in addressing fundamental evolutionary questions. What properties make RNA viruses excellent models for experimental evolution? First, as a consequence of the lack of proofreading of their RNA-dependent RNA polymerases (RdRp), they show mutation rates within the range $\sim 2 \times 10^{-5}$ to 1×10^{-4} per nucleotide per replication event (Drake and Holland, 1999); being variable is a key factor for pathogenicity. Second, genomes are small (for example, ranging from the 3.6 kb of bacteriophage MS2 up to the $\sim$30 kb of the coronaviruses), with many cases of overlapping reading frames or multifunctional proteins. Abundant molecular, functional, and structural information facilitates the analysis of the molecular basis of evolution. Third, replication rates are fast, and thus most RNA viruses reach very large population sizes after short periods of time. Plant viruses have the added value of making experiments with real hosts easy compared with experiments with animal viruses, which traditionally have been restricted to cell cultures due to the ethical and economical concerns inherent to the infection of large numbers of animals. Moreover, not only the evolution of the virus, but also the evolution of host–virus interactions, including the expression of virulence, can be studied in a natural framework. Finally, as opposed to experiments with bacterial viruses, plant viruses allow the study of complex eukaryotic multicellular hosts.

In this review, we will focus on recent studies that used plant viruses to address evolutionary questions of general interest, such as the rate and fitness effects of deleterious mutations and the role of neutrality as a source of mutational robustness, the evolution of generalist viruses, or the effect of vertical versus horizontal transmission on virulence. All these topics have also been explored using bacteriophages and/or animal viruses; to keep our review within bounds we shall not refer to this literature. Interested readers may take a look at the exhaustive review on experimental virus evolution by Elena and Sanjuán (2007).

Replication kinetics and mutation rate

As any other ribovirus that encodes an RdRp lacking proofreading activity, plant riboviruses have the potential to generate extremely variable populations. A recent study provided evidence for this high mutation rate. Malpica *et al*. (2002) infected *Nicotiana tabacum* plants, transgenic for the movement protein (MP) of *Tobacco mosaic tobamovirus* (TMV), with TMV and characterized

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the mutational spectrum for the replicating viral population at the MP locus. Since MP is provided *in trans* by the plant, the virus-encoded copy acts as a neutral marker wherein all sorts of mutations can be accumulated without affecting viral movement. The mutational spectrum was dominated by insertions and deletions, with only 31% being single nucleotide substitutions and with transitions and transversions arising at approximately the same rate. In nature, insertion/deletion mutations would be almost immediately lethal, hence explaining why these are found to be typically rare in natural variability studies. The genomic mutation rate, U, inferred was in the range 0.043–0.063 per replication event. However, if these estimates were corrected to account for a less efficient rescue of lethal mutants (Malpica *et al*., 2002), they would become twice as large ($\sim$0.1), which although still high, is approximately sevenfold lower than the estimates previously reported for lytic riboviruses (Drake and Holland, 1999).

Despite mutation rate is still high compared to that of DNA-based microorganisms, a classic field observation is that natural plant virus populations generally exhibit limited genetic variation (García-Arenal *et al*., 2001), which may imply either that purifying selection may be strong or that genome replication occurs mainly by Luria's stamping machine model (Luria, 1951) rather than exponentially (French and Stenger, 2003), the two hypotheses being nonexclusive. For TMV, a positive strand virus, the RdRp copies the first positive strand into several negative strands that are used as templates to produce multiple progeny-positive strands that are encapsidated to form new virions. The bulk of positive strands are synthesized in a linear process. The implications of the linear versus exponential growth are relevant to understanding the genesis of variability. In contrast, under the stamping machine model the frequency of mutation-free genomes among the N produced within an infected cell is given by the Poisson null class e^{-U} whereas for exponential growth this frequency depends on k, the number of replication events required to produce N viral genomes per cell, e^{-kU}. Thus, exponential replication produces $(1-e^{-kU})/(1-e^{-U})$ more mutant genomes per infected cell. Using the above estimates of U for TMV and assuming that $N=40$ per cell (Malpica *et al*., 2002), 339–350% more mutant genomes would be produced if replication was purely exponential. On the other hand, exponential replication has the advantage of being faster. Therefore, a tradeoff exists between the necessity of replicating faster and that of maintaining mutational load at as low a level as possible. It has been postulated that, since lysis is not the final fate of infected plant cells but instead viral particles accumulate in the cytoplasm forming densely packed bodies, selection for shorter lysis times may have been relaxed, thus giving advantage to a stamping machine replication mode (French and Stenger, 2003).

Genetic drift and viral movement

Genetic bottlenecks unavoidably occur at two different stages during the infectious cycle of plant viruses. First, it has been extensively shown that bottlenecks take place during plant virus systemic movement between leaves of the same plant, the number of viral particles being propagated on the order of tens or less for TMV (Sacristán *et al*., 2003) and *Cucumber mosaic cucumovirus* (CMV) (Li and Roossinck, 2004) or as low as four for *Wheat streak mosaic tritimovirus* (WSMV) (French and Stenger, 2003). Furthermore, these bottlenecks contribute to the establishment of genetically divergent populations in different parts of the plant and the generation of patched viral populations (French and Stenger, 2003; Jridi *et al*., 2006). Second, during aphid-mediated plant-to-plant transmission, strong bottlenecks have also been reported for CMV (Ali *et al*., 2006). Apparently, the bottleneck was not only due to the sampling of the donor plant during aphid feeding but also to the inoculation of the receiving plants (Ali *et al*., 2006).

The fitness effect of deleterious mutations

Associated with the stochastic nature of the aforementioned transmission or tissue colonization events, viral populations are continuously regenerated from a few individual genomes, and given the high mutation rate, a nonnegligible probability exists that deleterious mutations become fixed in the new population. Repeated genetic bottleneck events followed by low-fidelity genome replication have been shown to produce substantial fitness losses in several RNA viruses, in a process similar to Muller's ratchet (Elena and Sanjuán, 2007).

Recently, de la Iglesia and Elena (2007) have extended these observations to *Tobacco etch potyvirus* (TEV). In this experiment, bottlenecks of size one were performed by inoculating the nonsystemic host *Chenopodium quinoa* and by isolating the viral progeny contained in one randomly chosen local necrotic lesion. Local necrotic lesions, similar to what happens in the lytic plaques formed by bacteriophages in a bacterial loan, are generated from a single infectious unit. Figure 1 shows the dynamics of fitness decline for the independent mutation accumulation lineages evolved in this experiment. After 11 consecutive transfers, all lineages suffered significant fitness losses, and one became extinct. The per day average fitness decline was $U_d\bar{s}=5.2\%$, where U_d stands for the deleterious genomic mutation rate per transfer

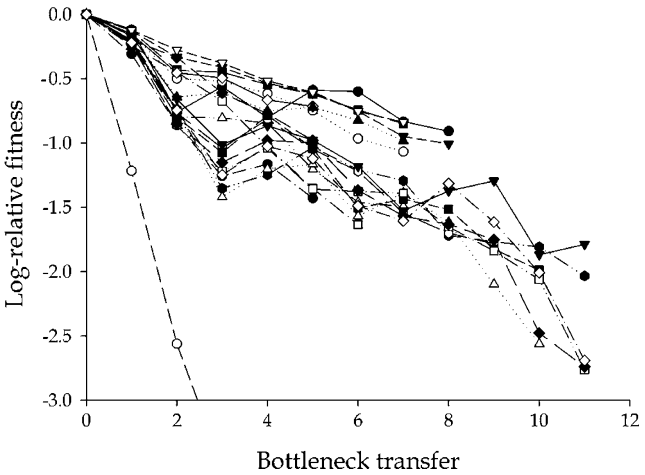

Figure 1 Evolution of TEV fitness upon serial bottleneck transfers. Each symbol and line represents a different mutation accumulation lineage. Fitness was expressed as the ratio between the Malthusian parameter estimated for each clone and the value obtained to the ancestral TEV. (From de la Iglesia and Elena (2007)).

Table 1 Proportion of lethal, deleterious, and neutral effects on fitness

	Proportion (%)	*Average effect (%)*
Lethal	40.9	−100
Deleterious	36.4	−51.1 ± 4.3
Neutral	22.7	

[From Carrasco *et al*. (2007b)].

and $\bar{s}$ for the average mutational effect. In a similar experiment with WSMV, a phylogenetically related virus, Hall *et al*. (2001) observed that the number of fixed mutations increased with bottleneck passages. Therefore, if purifying selection is not efficient because of small population size, the mutations accumulated are mostly deleterious.

Although informative, mutation accumulation experiments suffer from the problem that the number of mutations fixed per clone and its molecular nature remain unknown and the fraction of lethal mutations is undetectable. Therefore, inferences are only possible for the distribution of accumulated deleterious (but viable) effects. A straightforward way to avoid this problem is to create a collection of single-nucleotide substitution mutants by site-directed mutagenesis on an infectious cDNA and then measure fitness for each member of the collection to infer the statistical properties of the distribution of mutational fitness effects. This approach was recently undertaken by Carrasco *et al*. (2007a, b) to explore the distribution of mutational effects on the fitness of TEV. These authors created a collection of 66 clones each carrying a different, randomly chosen, single nucleotide substitution. The fitness of each clone was measured by competition experiments against a surrogated wild type. Table 1 shows a summary of the results of this experiment. Most mutations were lethal, with nonlethal mutations causing $\bar{s} = 51\%$ fitness reduction. Given the structural compactness and functional nonredundancy of RNA viruses, it is not unexpected that most mutations will either be lethal or have a strong deleterious fitness effect.

Combining the estimate of $U_d\bar{s}$ obtained by de la Iglesia and Elena (2007) and the estimate of $\bar{s}$ obtained by Carrasco *et al*. (2007b), it is possible to infer an estimate for TEV deleterious mutation rate as $U_d = 0.1$, a value that is consistent with the one reported for TMV (Malpica *et al*., 2002).

Neutrality and mutational robustness

When the mutation rate is so high that most newly generated individuals are genetically contaminated, as it appears to be the case for RNA viruses, fitness can be maximized by reducing the impact of mutations on fitness, that is, making organisms more robust, even at the cost of a lower replication rate (van Nimwegen *et al*., 1999; Wilke *et al*., 2001). However, whether robustness is an evolved property or intrinsic to genetic systems is largely unknown, because the mechanistic basis for robustness would depend on the peculiarities of each system (Elena *et al*., 2006). One of the simplest situations in which mutational robustness evolves in a population is represented by the quasi-species model of molecular evolution (Schuster and Swetina, 1988). In a molecular quasi-species, selection and mutation interact in a subtle way: selection should maximize the average replication rate of the swarm of genotypes interconnected by mutation rather than favoring any one genotype that has the fastest replication. However, since a faster replication also implies the production of more mutant genomes with lower fitness, as a consequence, mutation would act as a selective agent that shapes the entire genome so that it becomes robust with respect to mutation. In other words, populations would move on genotypic space toward regions of higher neutrality (van Nimwegen *et al*., 1999). Thus, in a highly mutagenic environment, a quasi-species occupying a low but neutral and highly connected (that is, flat) region in the fitness landscape should outcompete a quasi-species located at a higher but narrower fitness peak in which most surrounding mutants are unfit (van Nimwegen *et al*., 1999; Wilke *et al*., 2001). This phenomenon has been called the quasi-species effect or, more recently, the survival of the flattest (Wilke *et al*., 2001), and despite its interest, it lacked strong empirical support.

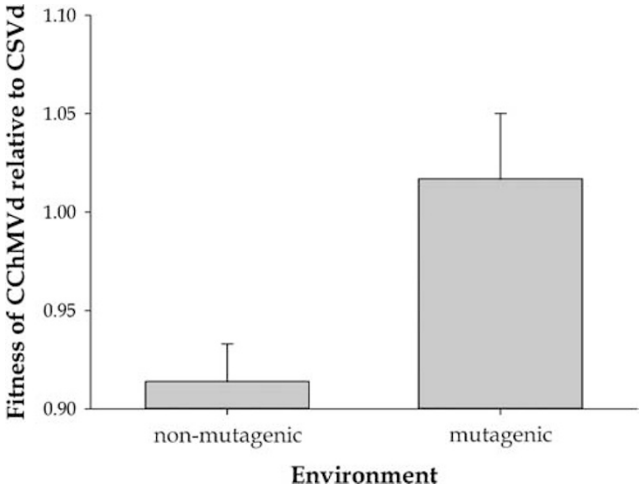

Figure 2 Fitness of a robust viroid, CChMVd, relative to a fit one, CSVd, in non-mutagenic and in mutagenic environmental conditions. Error bars represent standard errors. (From Codoñer *et al*., 2006).

Codoñer *et al*. (2006) used two viroid species; subviral plant pathogens whose genomes are constituted by a single small noncoding RNA molecule, to test this prediction. These two viroid species, which belonged to different families and are characterized by different secondary structures, competed in the same host plants. One of the viroids, *Chrysanthemum stunt pospiviroid* (CSVd), is characterized by fast population growth and genetic homogeneity, whereas the other, *Chrysanthemum chlorotic mottle avsunviroid* (CChMVd), shows slow population growth and a high degree of variation. Furthermore, the *in silico* size of the neutral neighborhood, that is, the number of point mutations that do not affect the predicted RNA folding, for CChMVd was found to be twice as large as that of CSVd. As expected, under standard nonmutagenic infection conditions, the faster replicator CSVd outcompeted the slower CChMVd, being the fitness difference of ~9% per week (Figure 2). However, as predicted by the quasi-species theory, when coinfected plants were irradiated with

mutagenic doses of UVC light, CSVd was unable to outcompete the slower but more robust CChMVd (Figure 2), suggesting that the competitive advantage of CSVd vanished as a consequence of the twofold larger mutational neutral neighborhood surrounding CChMVd's optimum genotype.

Host range and the specificity of adaptation

Broadly speaking, plant viruses have highly variable host ranges: some infect only one or a few related species (that is, they are specialists) whereas others can infect a wide range of hosts from different taxonomic groups (that is, they are generalists). The advantages of generalism are not well understood; it has been suggested that selection should favor specialist parasites because there are tradeoffs limiting the fitness of generalists in any of the alternative hosts or because evolution proceeds faster with narrower niches (Woolhouse *et al*., 2001). It is widely accepted that adaptation to a specific environment is often coupled with fitness losses in alternative environments simply because mutations that are beneficial in the first might be deleterious in the alternative (Kawecki, 1994). This antagonistic pleiotropy could limit the range of adaptation and promotes the evolution of specialization. In a recent field study, Malpica *et al*. (2006) found that five different plant viruses (from four viral families) were unevenly distributed across 21 weed species, some viruses being more prevalent on a given host than on alternative hosts, regardless of the relative abundance of each host. These observations suggest that host specialization may be a successful strategy for plant viruses.

Many studies can be found in the literature in which the qualitative costs associated with viruses expanding their host range from sensitive to resistant plant genotypes had been explored. Jenner *et al*. (2002) went a step farther and quantified the fitness penalty on wild-type plants paid by *Turnip mosaic potyvirus* (TuMV) after expanding its host range from wild-type turnips to plants bearing the *TuRB01* resistance gene. The fitness of three resistance-breaking (that is, generalists) TuMV genotypes, CZE1, CDN1, and v35Tunos + 5570A > G, was evaluated on wild-type turnips by multiday competition assays against the wild-type specialist isolate UK1. The fitness costs associated with host range were 32.36% for CZE1, 53.91% for v35Tunos + 5570A > G, and 100% for CDN1. These results support the idea that whatever mutation confers the ability to infect a new host has a strong pleiotropic effect on the ancestral host.

One of the most remarkable examples of fast host-specific adaptation is the convergent evolution of *Pelargonium flower break carmovirus* (PFBV) populations adapted to *C. quinoa* (Rico *et al*., 2006). PFBV isolates maintained for a long time on quinoa leaves, by the staff of German Collection of Microorganisms and Cell Cultures, have five specific noncontiguous amino acid substitutions in the coat protein (CP), which were not present in other natural isolates. The quinoa-specific pattern of amino acids at the relevant sites is VFYII. When a Spanish isolate from geranium, with the wild-type pattern ASHMV, was mechanically inoculated on quinoa leaves, the viral population generated soon after the first passage had already fixed two of the quinoa-specific changes (ASYMI), and only four serial passages were necessary to fix the entire VFYII quinoa pattern (Rico *et al*., 2006). The fact that this pattern has never been found in the natural host, even partially, shows the heavy burden it may impose for viral fitness on geranium (Rico *et al*., 2006).

Several studies have also reported that the amount of virus genetic diversity is driven by constraints imposed by the host in as much as it is driven by its own genomic properties. For example, the amount of genetic variability in evolving CMV and TMV populations remained close to an equilibrium value throughout serial passages in a constant host (Schneider and Roossinck, 2000, 2001). However, variability was severely reduced after transferring the populations to new hosts, suggesting the existence of a bottleneck imposed by the new host, and subsequently. Variation was subsequently regenerated *de novo* and eventually reached a new equilibrium, which was characteristic of the new host and different from the ancestral one (Schneider and Roossinck, 2000, 2001).

Mode of transmission and virulence

Current theories for the evolution of virus virulence suggest that natural selection may favor a wide range of virulences depending on the mode of virus transmission (Ewald, 1994). When a virus is vertically transmitted (that is, from host parents to offspring), a reduction in virulence is expected because viral fitness is tightly linked to host reproductive success: the longer and healthier the host lives, the more offspring it may deliver (Lipsitch *et al*., 1996). By contrast, when the mode of transmission is mostly horizontal (that is, between unrelated hosts), selection would favor those viral strains with increased infectivity even at the cost of being more virulent (Lipsitch *et al*., 1996). Testing these predictions has been difficult because it requires having a parasite with both modes of transmission. This problem was overcome by Stewart *et al*. (2005) using *Barley stripe mosaic hordeivirus* (BSMV), a virus capable of infecting barleys, both horizontally and vertically. After four generations of horizontal transmission, on average, the virulence (for example, reduction in lifespan production of viable seeds) of BSMV increased ~166% (Figure 3a) and the infectivity increased ~78% (Figure 3b). In addition, a significant positive correlation was observed between virulence and infectivity, as hypothesized. By contrast, after three generations of vertical transmission, BSMV populations were ~68% less virulent (Figure 3a) but ~12% more efficient at transmitting vertically. These results clearly support the above expectation that virulence depends on the relative opportunities for horizontal versus vertical transmission. Two more interesting findings can be drawn from this study. First, as BSMV evolved increased horizontal infectivity, the overall rate of vertical transmission declined (Figure 3b) and the rate of horizontal transmission increased, suggesting that it might not be possible to improve simultaneously both transmission routes (Stewart *et al*., 2005). Second, changes in virulence were uncorrelated with changes in virus accumulation within hosts, indicating that virulence was not a direct consequence of BSMV replication and accumulation but it may be mediated by complex plant responses.

The existence of a tradeoff between virulence and the rate of horizontal transmission has also been postulated

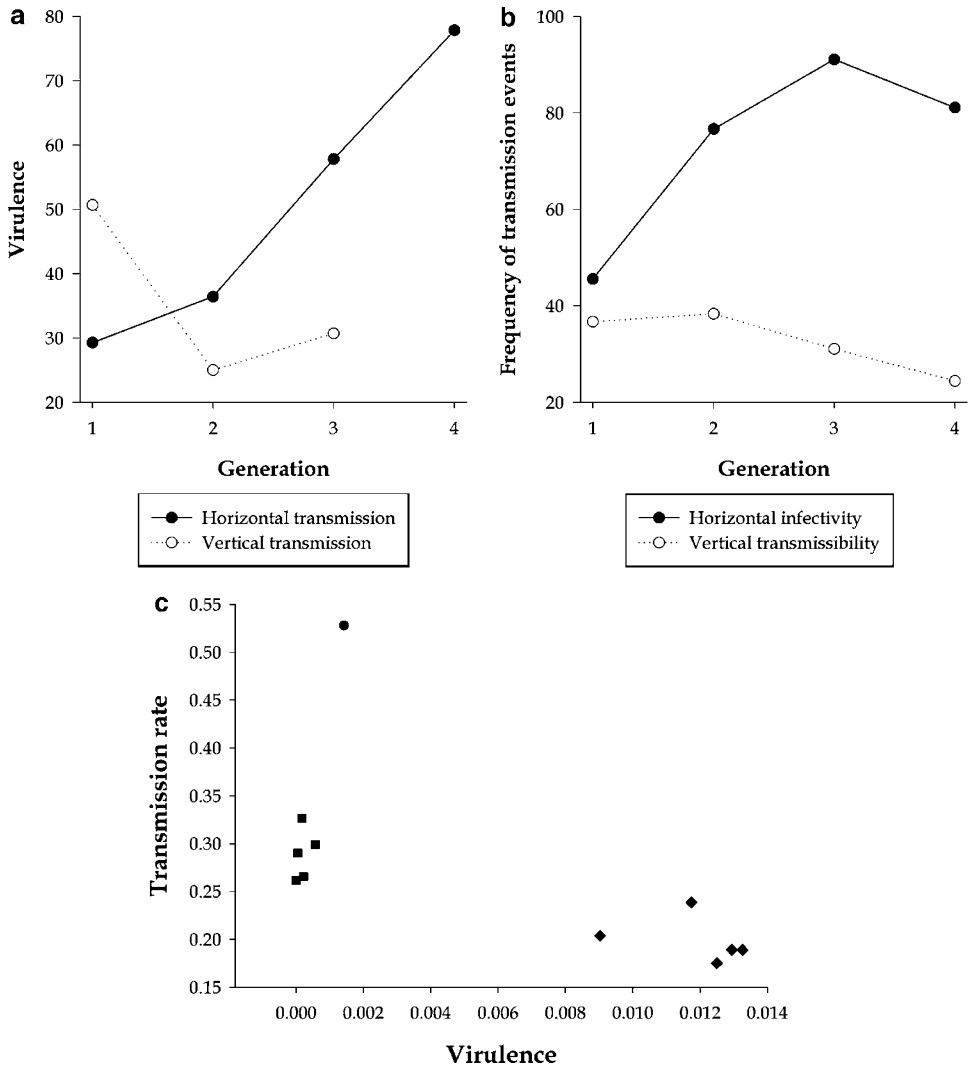

Figure 3 (**a**) Evolution of virulence in horizontally and vertically transmitted BSMV populations. (From Stewart *et al*., 2005). (**b**) Tradeoff between the efficiency of horizontal and vertical transmissions for BSMV populations evolved upon a horizontal transmission scheme. (From Stewart *et al*., 2005). (**c**) Correlation between virulence and transmission rate in CMV. Different symbols represent different CMV genotypes. (From Escriu *et al*., 2003).

(Ewald, 1994). The rationale for this tradeoff is that virus-induced host mortality shortens the period during which the virus can be taken up by vectors and transmitted from one host to another. Therefore, all else being equal, in a natural situation it may be costly for the virus to harm its plant host. To test this prediction, Escriu *et al*. (2003) performed field and laboratory studies seeking to understand the evolution of virulence (for example, reduction in lifespan biomass) and epidemiology of different CMV strains. The data gathered by these authors support the predicted negative correlation between virulence and the rate of transmission (Figure 3c: partial correlation coefficient controlling for strain type: $r = -0.96$, d.f. $= 8$, $P < 0.001$). In other words, in natural conditions, necrogenic CMV strains may jeopardize their own transmission by aphids by killing the plant, or making it less attractive to the vector insects, too quickly. However, in good agreement with the BSMV study, CMV virulence was not dependent on its accumulation level since necrogenic and nonnecrogenic variants accumulated to similar levels (Escriu *et al*., 2000), thus suggesting that virulence may be a side effect of the interaction between the virus and plant defense mechanisms.

Conclusions

In this contribution, we have briefly reviewed some recent advances in plant virus experimental evolution. Our goal was not to provide an exhaustive review of all the relevant literature but, instead, to illustrate the advantages of plant RNA viruses as model systems for experimental evolution studies. Traditionally, animal viruses and, in particular, bacteriophages have been widely used for testing evolutionary hypotheses, whereas plant viruses have received less attention. Here we have shown that the same questions can be also addressed using plant RNA viruses (for example, the pace and effect of deleterious mutations on fitness or the specificity of adaptation) but, in addition, new exciting questions have also been properly addressed (for

example, evolution of virulence in a natural host–parasite system and the role of neutrality on evolution).

Plant RNA viruses open promising new avenues for experimental evolution. Basic evolutionary problems such as the dynamics of adaptation and genetic diversification, the evolution of viral takeover of cellular metabolism, the evolution of virulence during dynamics of multiple infection, testing gene-for-gene coevolutionary models, the evolution of mutational robustness, and coevolutionary interactions between multiple viruses infecting the same host, among others, will be tackled in the near future using plant viruses.

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