Advances in Plant Virus Evolution: Translating Evolutionary Insights into Better Disease Management

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Virus Acronyms: *African cassava mosaic virus* (ACMV), *Beet necrotic yellow vein virus* (BNYVV), *Brome Mosaic virus* (BMV), *Cabbage leaf curl virus* (CaLCuV), C*auliflower mosaic virus* (CaMV), *Chrysanthemum chlorotic mottle viroid* (CChMVd), *Citrus leaf blotch virus* (CLBV), *Citrus tristeza virus* (CTV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing virus* (CVYV), *East African cassava mosaic virus* (EACMV), *Mung bean yellow mosaic virus* (MYMV), *Oilseed rape mosaic virus* (ORMV), *Plum pox virus* (PPV), *Potato virus X* (PVX), *Potato virus Y* (PVY), *Rice yellow mottle virus* (RYMV), *Soil-borne wheat mosaic virus* (SBWMV), *Tobacco etch virus* (TEV), *Tobacco mild green mosaic virus* (TMGMV), *Tobacco mosaic virus* (TMV), Turnip *mosaic virus* (TuMV), *Turnip vein clearing virus* (TVCV), *Wheat streak mosaic virus* (WSMV).

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ABSTRACT

Recent works in plant virus evolution are revealing that genetic structure and behavior of virus and viroid populations can explain important pathogenic properties of these agents, such as host resistance breakdown, disease severity, and host shifting among others. Genetic variation is essential for the survival of organisms. The exploration of how these subcellular parasites generate and maintain a certain frequency of mutations at the intra- and inter-host levels is revealing novel molecular virus-plant interactions. They emphasize the role of host environment in the dynamic genetic composition of virus populations. Functional genomics has identified host factors that are transcriptionally altered after virus infections. The analyses of these data by means of systems biology approaches are uncovering critical plant genes specifically targeted by viruses during host adaptation. Also, a next-generation re-sequencing approach of a whole virus genome is opening new avenues to study virus recombination and the relationships between intra-host virus composition and pathogenesis. Altogether, the analyzed data indicate that systematic disruption of some specific parameters of evolving virus populations could lead to more efficient ways of disease prevention, eradication, or tolerable virus-plant coexistence.

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Viruses and viroids appear to be the fastest-evolving plant pathogens (39), and cause tremendous economical crop losses annually. Some, such as the single-stranded DNA begomoviruses, are emergent problems worldwide (117, 126). These subcellular pathogens have higher mutation rates than, and distinct evolutionary dynamics from, bacterial and fungal phytopathogens. Understanding their reproductive and transmission strategies – their biology, ecology, and evolution – can lead to insights and interventions for effective crop disease management. This review highlights how viruses and viroids achieve and maintain their unique parasitic lifestyles and how evolutionary virology and systems biology approaches to virus-plant interactions have implications for pathogen control.

The ability of viruses and viroids to change, and to change rapidly, underlies many disease management concerns. Excepting migration from distant locations and other countries, variability in plant pathogen populations is the necessary initial step in adaptation to new plants (host shifting), resistance breaking (RB), and changes in symptoms and virulence. Many times the rise and fall of different genotypes in a population is due to the effects of natural selection: variant genomes that generate more viable descendants become more frequent over time. This process can be sped up or inhibited by **bottlenecks** (see Textbox 1 for definitions of terms in bold), that plant pathogens experience as they move from cell to cell and from plant to plant (57, 69, 88). To expand its host range, a virus population must already have a variant (perhaps at a very low level in the population) that can infect that potential host. In the novel host, those mutants will be fitter and will rise in frequency. However, in absence of selection on that novel host, the only chance that neutral **host range mutations** have to be fixed in the population is by **genetic drift**. Otherwise, neutral or even deleterious mutations will be sweep away by **purifying selection**. Understanding the processes that generate viral diversity and the ecological processes that determine selective pressures and bottlenecks can illuminate potential interventions or determine where and when control measures might be most effective.

Mechanisms of virus and viroid genetic variation. Viruses have several ways to achieve variation within a plant and within a field. First and foremost is mutation: the imperfect copying of genomic material from parent to offspring and subsequent chemical and enzymatic changes to nucleotide bases. RNA viruses are notorious for having high **mutation rates**, due to replication with RNA-dependent polymerases lacking proofreading activity. The polymerases of large nidoviruses are an exception (100), but none have yet been shown to infect plants. Most studies of plant virus variation measure **mutation frequencies** over a range of time, such as viral mutations arising within a month after plant infection. These studies are popular in plant virology because of the difficulty in relating mutation frequency to how often the viruses have replicated their genome within a whole plant. Despite this limitation, mutation frequencies can be used to estimate upper boundaries on plant viral mutation rates (reviewed in 120). Mutation rate studies tally the mutations produced prior to the action of selection, either per round of genomic replication or per cell infected. Two exceptional studies have calculated mutation rates: one for TMV (94) and the other for TEV (135). In both cases, the mutation rates trend towards the lower end of measured animal and bacterial RNA virus rates (Fig. 1).

Plant RNA viruses may indeed have lower mutation rates than animal RNA viruses, but the existing data show that they are not substantially lower, and some of the differences could result from methodological dissimilarities. In fact, some plant pathogens do mutate faster than animal RNA viruses: plant viroids, for instance, have the highest per-base mutation rate yet measured for any disease-causing agent at 2.5×10^{-3} per base per round of replication (61) (Fig. 1). Despite this highest per base mutation rate, viroids seem to obey the constant of around one mutation per replicated genome reported by Drake et al., (36) for most RNA viruses. This fairly constant per-genome mutation rate suggests that RNA genome sizes are limited by their individual per-base mutation rates. Otherwise, a larger genome replicated with a given per-base mutation rate, for example, a closterovirus with a per-base mutation rate of a viroid, would be

unable to maintain functional elements because of the accumulation of too many mutations in its genes. Thus, a population of such larger genomes would collapse due to **lethal mutagenesis** (17). Recombination may play a significant role in the virus survival by reducing the amount of deleterious mutations incorporated in the same virus genome.

Viroids are the smallest known pathogenic agents of plants and cause diseases of considerable economic importance (33, 34). Viroid genomes are composed of a single-stranded, self-complementary RNA molecule of 246 to 475 nucleotides. Viroids lack the capacity to code for proteins, are not encapsidated, and are replicated by host-encoded polymerases (32). They are classified into two families: those that replicate in the nucleus (*Pospiviroidae*) and those that replicate in the chloroplast (*Avsunviroidae*) (29). The most abundant Pospiviroids are characterized by the presence of a central conserved region, absence of hammerhead ribozymes, and nuclear replication via an asymmetric rolling circle by a nuclear DNA-dependent RNA polymerase II. Avsunviroids are characterized by the absence of a central conserved region, the presence of a hammerhead ribozyme, and replication in the chloroplast via a symmetric rolling circle by another nuclear-encoded chloroplast DNA-dependent RNA polymerase. Whereas pospiviroids are predicted to be primarily rod-shaped, avsunviroids are predicted to be more highly branched structures. Thus, viroid molecules are a collection of structural-sequence motifs that interact with host components for viroid replication, processing, transport, and pathogenesis that may all influence viroid evolution (153). Avsunviroids populations appear to have more haplotypes than pospiviroids (26). It has been estimated that the mutation rate of avsunviroids is 10-fold larger than for pospiviroids (41, 46). Apparently, most mutations in the rod-like structure of pospiviroids are deleterious. In fact, many stable mutations of viroid genomes map in loops or as compensatory mutations in hairpins and stems (8, 30).

Therefore, how viroids achieve the highest mutation rates among the known infectious RNAs observed in nature is still a matter of speculation. Viroids are replicated by DNA-dependent RNA polymerases with variable proofreading efficiencies. Even more, when these enzymes use RNA instead of native DNA as template, their replicative fidelity could be further reduced. Another putative mechanism, by which their mutation rates could be elevated above that of the polymerase error, and it could also happen to viral genomes, is through enzymatic changes to nucleotide bases. Cytidine deaminases are enzymes that turn cytidine (C) into uridine (U), and mammals use a family of them (APOBEC) as anti-viral defense against some animal viruses (73). Plants have orthologous proteins that have known roles in post-transcriptional modification, and they are most active in mitochondria and chloroplasts (24). It may be the case that these plant enzymes can be active on virus and viroid RNA as well, which would increase C→U substitution rates. Other deaminases increase substitutions such as A→I (adenosine to inosine). Patterns of frequent cytosine deamination have also been detected during ssDNA geminivirus evolution (37). This could be due to these enzymes reacting with single-stranded DNA viral genomes, but C→U is also the most common kind of spontaneous chemical degradation that can occur on unpaired nucleotides so cytosine transitions could be increased solely because geminiviral DNA is frequently single-stranded.

Substantial virus and viroid diversity is generated in plants also by homologous and heterologous recombination. Pathogens often co-infect the same plant, allowing co-infection of single cells (102), and some viruses frequently take the opportunity to unequally exchange genes (23, 66, 87). While recombinants mostly appear to be tolerated between conspecific viruses, intergenus recombination is also possible, and recombination can even lead to incorporation of host genetic material. Recombination rates are difficult to measure, but could be as high as mutation rates: BMV could exhibit an homologous crossover event per RNA molecule per replication cycle (139), CaMV has an estimated recombination rate of around

170 $2x10^{-5}$ per round of replication (59). It has been proposed that recombination may have led to
171 the emergence of mosaic sequences from viroids co-infecting the same host (74, 106).
172 Recombinants have been associated with altered host range and virulence (107). One clear
173 example of a virulent recombinant was a hybrid of EACMV and ACMV that overcame crop
174 resistance to ACMV and decimated cassava production in Uganda in 1997 (113).

175 ***Plant viruses evolve quickly***. The speed of evolution can be estimated by the rate
176 at which the genetic makeup of a population changes in time by selection and/or genetic drift.
177 Representatives of the RNA families *Potyviridae* (67, 127), *Tobamoviridae* (108), and a
178 sobemovirus (52), and the ssDNA families *Geminiviridae* (37, 38, 75) and *Nanoviridae* (70)
179 evolve faster than 10^{-5} fixed nucleotide substitutions/site/year (s/s/y), and as high as 10^{-3} s/s/y.
180 There does not appear to be a distinction between the evolution rate of plant versus animal
181 viruses (39, 65), and between RNA and ssDNA viral substitution rates in plants: all plant viruses
182 appear to be fast-evolving.

183 These results were initially at odds with the plant virus evolution literature, which
184 stressed overall genetic stability over time, especially in comparison to viruses of animals with
185 adaptive immune systems (63, 66, 149). Indeed, substantial purifying selection exists for plant
186 viruses to maintain nucleotide or amino acid sequence that reduce nucleotide substitution rates.
187 One well-established source of purifying selection is the alternation in selective pressures that
188 vectored viruses experience when cycling between animal and plant cells. The capsid proteins
189 of these viruses, which interact with both host and vector, are under more pressure to be
190 unchanging, as evidenced by very low ratios of the rate of nonsynonymous changes to the rate
191 of synonymous changes (d_N/d_S) (22). This purifying selection also leads to lower average rates
192 of capsid gene evolution in vectored animal viruses than in directly transmitted viruses (79). Still,
193 the measured and estimated high nucleotide substitution rates of plant viruses occurs in
194 presence of purifying selection, and the evolutionary rate of vectored plant viruses is within the

same order of magnitude to some directly transmitted viruses. But an important point to stress is that these rates of evolution are usually calculated over many years, if not decades, and reflect an average nucleotide substitution rate. As will be shown in a subsequent section, rates of mutation fixation can be higher when viruses are under **positive selection**, such as when they are adapting to a novel host plant and several beneficial mutations may become ascendant in a short period of time.

Variability sometimes leads to adaptability. The average mutation and the average recombination event are deleterious, and many are lethal. Around 70% of mutations are deleterious (20, 35, 122). Consequently, variants that are produced are not always maintained in viral populations. However, some of those changes that are deleterious in the current host and environment may be adaptive under different conditions (40, 53). Mutations that are deleterious in the current host will be maintained in a population if they are either mildly deleterious (nearly-neutral), if they are continually created by new mutational events, or at a ratio of these two factors called the **mutation-selection balance**.

The mutation-selection balance is one, but not the only factor determining whether a mutation will be part of a virus population. **Complementation** between co-infecting viruses can result in viral genomes carrying deleterious, even lethal, mutations to be maintained in populations (60). Plant viruses can even develop defective interfering genomes that require at least one essential component or function be supplied in *trans* by another virus genome (90). Viral proteins that generally are shared among particles are involved in coating, cell-to-cell movement, and suppression of gene silencing, but many other proteins may operate in *trans* exclusively in some virus species. Defective interfering genomes are distinct from satellite viruses, which rely on complementation but do not descend from the helper virus. Many satellites attenuate the effects of the primary virus infection (128), but a minority can increase virulence, such as the beta satellites of ssDNA begomoviruses (16). Similarly, defective

220 interfering genomes modulate infection severity, often competing with the complementing virus
221 and reducing virulence (111, 128).

222 Maintaining population variability is what allows for the chance encounter between a
223 novel environment and a mutation that is beneficial in that environment, such as a host range
224 mutation. However, high variability does not necessarily indicate great adaptive potential. For
225 instance, genetic **robustness** is based on a number of buffering mechanisms (including genetic
226 redundancy and cellular chaperones) that minimize mutational effects (44). Since plant viruses
227 and viroids mutate so frequently, and the vast majority of mutations are deleterious, robustness
228 is often a successful evolutionary strategy. The empirical demonstration of robustness operating
229 in an infectious RNA was provided by Codoñer et al. (26). They designed a co-infecting
230 competition experiment for this purpose. In this experiment, slow replicating and highly
231 heterogeneous populations of an avsunviroid outcompeted fast replicating and relatively
232 homogeneous pospiviroid populations only when the mutation rates of both viroids were
233 artificially increased by UV irradiation. Thus, under such conditions, the highly heterogeneous
234 viroid populations were more adapted. Whether the increased robustness in the avsunviroid is
235 due to its more relaxed RNA secondary structure or its higher genetic heterogeneity is still
236 unknown.

237 Eventually, even robust genomes will show effects due to the numerous mutations
238 accumulated in their genomes, but it is in unpredictable ways. The accumulated mutations may
239 together cause large drops in fitness, even though some individual genotypes may be adaptive
240 in the current host (18). Additionally, robustness is often specific to a virus in a particular
241 environment, shifting to another host can cause plant viruses to be less robust, and show the
242 effects of their accumulated genetic diversity and subsequent mutations. The relationship
243 between robustness and the ability to evolve and adapt, is complex and murky (99). Further
244 complicating the practical interpretation of viral and viroid genetic diversity, robustness does not

explain all examples where greater variability fails to lead to greater adaptability. Despite the greater robustness of an avsunviroid mentioned above, members of this family generally infect fewer hosts and appear to host-shift less often and have more restricted host ranges than pospiviroids (41). Therefore, more research is required into the evolution of a wide variety of plant subcellular pathogens to understand the interplay between genetic heterogeneity of the population and adaptation.

EPIDEMIOLOGICAL DYNAMICS OF VIRUS POPULATIONS

A premise in virus evolution is that the dynamic genetic structure of virus populations has a significant role in virulence, epidemiological progression of the disease, and host shifting among other biological properties of viruses (76). The mechanisms that regulate the genetic structure (i.e., number and frequency of haplotypes, and genetic distances among them) of virus populations and their biological relevance are presented in this section.

Dynamics of the INTRA-host genetic structure of plant virus populations. Theoretically, the structure of an active virus population changes within a host individual during the course of a systemic infection. Despite the random emergence of spontaneous mutations, these structural changes could exhibit deterministic or stochastic behavior (118). The former has been observed primarily in compatible virus-plant interactions (i.e., between virulent pathogen and susceptible host), whereas stochastic population structures are more frequently generated in incompatible interactions (i.e., avirulent pathogen and resistant host) or during viral host adaptation. Intra-host populations of TMGMV, despite their heterogeneous composition, exhibit high genetic stability in field infections of *Nicotiana glauca* (55, 103, 115). High genetic stability also appears to be the norm in several other plant viruses infecting their compatible hosts (reviewed in by 63). This relative genetic stability suggests that

virus populations might not undergo substantial changes while they are interacting with their natural host genotype. Purifying selection affecting the d_N/d_S ratio in the order of 0.01 to 0.31 has been found to operate in highly adapted virus populations, maintaining them in equilibrium (62). The mutation frequency of plant viruses are influenced by host species (124). Recently, a comparative bioassay of BNYVV infecting compatible versus incompatible host genotypes demonstrated that more variability exists in small BNYVV populations from partially resistant than in large populations produced in susceptible hosts (1). These data agree with the high genetic stability of BNYVV prevailing worldwide during long periods in susceptible sugar beet cultivars and the sudden stochastic diversification of BNYVV observed after the deployment of resistant genotypes in the field (2, 85). Similarly, for other plant viruses, higher diversity has been recorded at their centers of origin, where the plant virus presumably initiated its adaptation to a new host (51, 104, 133).

Without external input variation by superinfections, the increase in the intra-host genetic diversity that some plant virus populations experience under restrictive host conditions is most likely the result from deviations of the mutation-selection balance. Accumulating evidence indicates that mutation rate, rather than an invariable property of the virus, may fluctuate in response to changes in viral replicase and specific cellular conditions (39, 47). For instance, the terms mutators and antimutators have been coined for individuals within a species that have an inheritable higher or lower mutation rate than the wild type, respectively (95). They have been discovered in bacteria, bacteriophages, and human viruses (reviewed in 129). Individuals encoding each one of these mutational phenotypes might coexist in the same intra-host population and, under some conditions, one will eventually predominate. Although neither a plant virus nor a viroid isolate has exhibited mutator or antimutator phenotypes, greater than usual mutation frequencies have been detected for PVY and BNYVV only under restrictive host environments (1, 4).

Once mutations have been introduced in a virus population, their frequency is regulated by their interaction with other existing mutations. Generally speaking, the interaction between genetic loci is known as **epistasis**. Epistasis can be antagonistic or synergistic depending on its effect on fitness (76). Antagonistic epistasis means that two deleterious or beneficial mutations carried in a given genome can be, respectively, not as bad or as good in combination as expected by combining their individual fitness effects. Synergistic epistasis, on the other hand, enlarges the total fitness effect of the interacting mutations. Given the extreme genome compactness, gene overlapping, and lack of genetic redundancy, most mutations in virus genomes show antagonistic epistasis (20). Coincidently viral genome architectures that favor antagonistic epistasis are less robust to spontaneous mutations than those with greater synergistic epistasis (44). *Trans* complementation and **interference** also regulate the frequency and prevalence of mutants into the infecting virus population (60). The genetic structure that results from the combined action of all these interactions, rather than specific genotypes composing the population, could be the target of selection.

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Comentario [1]: Creo que así queda un poquito más claro.

Dynamics of the INTER-host diversity of virus populations. The largest amount of genetic data available on plant virus populations is for the inter-host level. The data could be from individual or pooled plant samples and, typically, it is represented by consensus sequences of a specific region of the virus genome. One of the most striking observations derived from these data is the apparent differentiation between fully host-adapted and host-adapting virus populations. In both of these types of compatible virus-plant interactions, viruses reach high titers in infected plants, but in host-adapted populations the genetic diversity among isolates is several orders of magnitude lower than in host-adapting populations. The genetic diversification of WSMV can be used as a reference point for the rate of plant virus evolution. Phylogenetic analysis of 54 WSMV field isolates from North America suggests that they arose from a common ancestor introduced nearly a hundred years ago. The number of segregating

polymorphic sites in this representative population was 0.047 and the mean pairwise **nucleotide diversity** (**π**) 0.020 (130). Phylogeographic studies of RYMV infecting rice crops in Africa pinpointed the center of origin of RYMV to eastern Tanzania from where it migrated northwest up the west coast of Africa (51). The largest RYMV inter-host π per Km^2 occurs in Tanzania (i.e., around 200 times greater than in any other region of the continent) without a correlation with the diversity of its potential host species (114). Therefore, it is likely that the high RYMV diversification in Tanzania may have resulted from disruptions of the mutation selection-balance that initially took place at the intra-host level and then among adapting isolates. Similarly, around 240 times greater BNYVV nucleotide diversity was detected between RB variants recently emerging in the Imperial Valley of California (π = 0.0024) than wild type isolates collected nationwide (π = 0.00001) (3). Other factors that could be correlated with the magnitude of inter-host virus diversity are the genetic diversity of the host, frequency of superinfections, transmission mechanisms, etc. However, other than greater virus diversity detected in sexual than asexual host genotypes (105) the effects that those variables may have on plant virus populations apparently have not been explored.

Relationships between INTRA- and INTER-host virus diversity. Except for those viruses that mechanical inoculation is part of their mode of transmission in the field (11, 116), limited data exists concerning the intra- and inter-host genetic structure of vector-transmitted virus populations. These are some of the few available examples where the population was not mechanically inoculated prior to the analysis, which may disturb the natural structure. The π value of whitefly-transmitted isolates of CVYV was around 0.0005 among clones from two single-plant populations and 3.4 times greater among 56 consensus sequences (78). Similarly, Vives et al. (142) found that the intra-host π of CLBV in 37 citrus trees naturally infected (unknown vector) in a region of Spain, was three to four times lower than the inter-host π. Theoretical analyses indicate that greater genetic diversities between (i.e., π_B) rather than

344 within (i.e., π_W) single-plant populations is favored by random genetic drift during virus
345 propagation (62). If genetic drift occurs more frequently at suboptimal virus fitness because π_W
346 is higher than at optimal fitness, then, it is expected that the π_W/π_B ratio of virus populations will
347 be related to virus fitness as described in Figure 2. In this way, the ratio π_W/π_B could reflect the
348 level of viral host adaptation. Initial support for this model has been obtained by comparing the
349 nucleotide diversities of BNYVV populations from composite samples of resistant *Rz1* sugar
350 beets (3). Pooled samples apparently are more reliable for this type of analysis because they
351 minimize the intrinsic plant-to-plant variation. This analysis revealed that, while BNYVV titers are
352 10^2 to 10^4 times higher in symptomatic plants infected by emerging RB variants than
353 asymptomatic plants infected by avirulent virus populations, the opposite occurs in relation with
354 the π_W/π_B ratio. It was 7.8 to 12.2 times larger in asymptomatic than symptomatic resistant
355 plants (i.e., π_W/π_B = 6.1 to 16.7 versus 0.5 to 2.1, respectively).

356 ***Population bottleneck as an additional modulator of genetic diversity***.
357 Sustaining variation is a particular challenge for plant viruses because severe genetic
358 bottlenecks exist as vectored viruses move from plant to plant and even within plants.
359 Population bottlenecks can occur during vector inoculation, cell-to-cell movement, vascular
360 access, vascular transport, vascular exit, specific tissue entry (i.e., lateral roots, endosperm,
361 meristems, etc.), vector acquisition, viruliferous vector migration, and alternate host infections
362 (57, 88). These conditions create spatial structure in how the virus diversity is distributed
363 throughout the plant and the field. Within each new plant or portion of a plant, a viral population
364 regains diversity as it multiplies from a small initial number of infecting genomes. After repeated
365 host-to-host transfers of WSMV, similar numbers of haplotypes were found whether one or two
366 strains initially infected the plant (58). Thus, bottleneck size during virus transmission is not
367 always correlated with the extent of regained variability in the derived population.

Throughout the life cycle of a plant virus, at any bottleneck event, the virus survival and its population structure are especially vulnerable. The **multiplicity of cellular infection** (**MOI**) has been developed as a way of estimating the number of virus genomes that invade a plant cell during the course of local and systemic virus infections. The first reported MOI of a plant virus was provided by González-Jara et al. (69). They estimated that approximately six TMV genomes initially infect *Nicotiana benthamiana* cells. Then, the MOI decreases to one to two genomes during the systemic infection process suggesting the involvement of mechanisms inhibiting superinfection at the advanced stages of the disease. Similarly, MOI of five to six genomes were estimated for SBWMV causing localized leaf infections in *Chenopodium quinoa* (101). MOI values around four, with a maximum of 13 during the acute phase of systemic infection, were calculated for CaMV infecting its natural host, *Brassica rapa* (72). In general, the estimation of cellular MOI requires viral genomes carrying specific neutral mutations or reporter genes to monitor the frequency and location of single and mixed infected cells. Newly generated mutant genomes that may have derived from the inoculated transcripts are not considered in these calculations, neither are virus genomes that may have been silenced or partially expressed. Therefore, MOI values, rather than being an absolute number, may represent a fraction of a larger and still unknown number of viral genomes per cell.

Theoretical analysis of the data presented above indicates that bottleneck size is critical in preserving the parental population structure including both adaptive and defective mutants. For instance, in virus populations where lethal mutants can only be maintained by *trans*-complementation by functional virus genes, the chances that these selfish mutants will predominate in the following generation are greater with broader bottlenecks because it increases the probabilities of co-infecting with fitted virus genomes (101). With narrow bottlenecks, on the other hand, only fit genomes will infect most of the cells and consequently the genetic diversity is expected to decrease by leaving behind defective mutants. For mutants

393 that can replicate by themselves, however, the opposite outcome is more likely, i.e., the genetic
394 diversity is expected to increase at smaller bottleneck sizes because low frequency neutral or
395 near neutral mutants have better chances of moving forward by random drift and be able to
396 infect neighboring cells by themselves. With broader bottlenecks, by contrast, the parental
397 population structure has more probabilities of being reproduced in most of the infected cells.

398 The impact that different levels of intercellular bottleneck may have on the genetic
399 structure of ensuing virus populations has not been empirically determined, but coincidental
400 data suggest that it could be extremely important for disease management. For instance, two
401 resistant tobacco genotypes with the same barrier to PVY cell-to-cell movement but different
402 levels of cellular virus accumulation exhibit drastic differences in the incidence of spontaneous
403 RB infections (4). The tobacco with the weak resistance durability, NC745, has larger PVY
404 populations in the initially infected cells than the near immune tobacco VAM. Consequently, the
405 proportion of particles moving from one cell to the next is smaller in NC745. Thus, although the
406 absolute number of moving viral particles might be the same in both tobacco genotypes the
407 proportions are not. This observation suggests that relative rather than absolute MOI values
408 could be a more reliable estimation of the effect of bottlenecking on plant resistance durability.

409

410 **THE SYSTEM BIOLOGY APPROACH**

411 Systems Biology (SB) allows assessing gene expression at a genome-wide scale,
412 providing unprecedented views of the virus-host interaction. SB deals with the study of
413 interactions between components of biological systems and how these interactions give rise to
414 the function and behavior of the system (14, 84).

415 To complete their infectious cycle, the few viral components must establish multiple and
416 complex interactions among them (54, 71, 89, 138) as well as with a large number of

components from the host (13, 132, 146). These interactions result either in the plant controlling the infection or in the virus overcoming defenses and establishing a systemic infection. Indeed, the recent application of the SB approach to the analysis of virus-host interactions has revealed a more complete picture of the sets of host factors required for virus infection (86, 146). Moreover, SB has uncovered highly connected host genes that operate as central elements in the plant regulatory network and are specifically targeted by viruses to control the host metabolism (19, 28, 42). Additionally, SB has evidenced topological changes of the intra-viral interaction network that are caused by its integration within the host network (93, 137).

While the SB approach has been increasingly used in the analysis of animal virus-host interactions (e.g. hepatitis C, human immunodeficiency, yellow fever, influenza A, or herpesviruses), plant virology has not yet benefitted to the same extent, and the most relevant studies in the field generally apply transcriptomic techniques to generate lists of genes with altered mRNA abundance in infected plants. However, the proper network analysis of virus-plant interaction is still a pending task (45).

Different viruses, common targets. Although some studies have analyzed changes in mRNA profiles resulting from viruses infecting their natural hosts, such as ACMV infecting cassava (56) or RYMV infecting rice (141), *Arabidopsis thaliana* has been extensively used as a model host in combination with viruses belonging to different taxonomic families (Table 1). However, even using the same host species, direct comparison across experiments is not straightforward because differences in profiling techniques and platforms, plant ecotypes, sampling schemes, inoculation conditions and dosages, and environmental variables may all exert some unpredictable effects on the expression pattern of multiple genes.

Whitham *et al*. (145) carried out the most comprehensive of such studies, including five viruses mentioned in Table 1 (CMV, ORMV, PVX, TVCV, and TuMV) while keeping all other

experimental variables and techniques constant. Some generalities were drawn from this study that are extensible to most of the other studies listed in Table 1. First, approximately one-third of over-expressed plant genes are associated with cell responses to situations of stress, defense against infection, apoptosis, programed cell death, and ageing. Second, defense-like responses of *A. thaliana* to viruses are dependent on salicylic acid (SA) and require upstream signaling components (148). Third, a spectrum of heat-shock proteins (HSP) is also induced after infection with all viruses by a yet unknown SA-independent mechanism. HSP over-expression may be a generic unspecific response of the plant to stress or, alternatively, directly triggered and controlled by viruses to assist the right folding of their own proteins, many of which may be misfolded (and thus aggregating) as a consequence of mutations produced during replication (81). Fourth, cell wall modification genes are preferentially down-regulated. Because the expression of these genes is correlated with plant cell growth and expansion, their reduced expression may well result in the stunting syndrome associated with some infections. Fifth, similarly, plastid genes and genes involved in chloroplast functioning are also preferentially down-regulated, resulting in chlorotic symptoms. Sixth, ribosomal proteins and protein turnover genes are up-regulated. This may either reflect an increased demand on the cells for protein synthesis or a response triggered by viruses to enhance its own replication.

Host-adaptation and changes in gene expression profiles. The actual interactions between viruses, natural hosts, and vectors are the results of natural selection operating during many generations. Hence, to have a precise description of the interactions established between viral and cellular components, it is necessary to take into account the evolutionary perspective of the process: the degree of adaptation of the virus to its host. Unfortunately, this evolutionary perspective has not been taken in most studies listed in Table 1: only TuMV, CMV and CaMV are prevalent in wild *A. thaliana* populations (109).

To test whether adaptation to a host may result in changes in mRNA profile, Agudelo-Romero *et al*. (6) performed an evolution experiment adapting TEV to the susceptible ecotype L*er*-0 of *A. thaliana* (25). The TEV clone used as the ancestral virus was able of systemically infect L*er*-0 plants although the infection was asymptomatic. After 17 serial passages, the resulting virus (labeled as TEV-*At*17) accumulated three orders of magnitude more than the ancestral one per gram of infected tissue, its infectivity was 100% (compared with the low 10% of the ancestral genotype) and induced severe symptoms including stunting, etching, and leaf malformation. A single amino acid substitution in the VPg was enough to trigger these symptoms. TEV-*At*17 infection caused the differential expression of a total of 505 up-regulated and 1335 down-regulated plant genes relative to its ancestor virus (Figure 3 in 6). Both viruses also differentially affected the expression of transcription factors, with 51 up-regulated and 84 down-regulated only by the evolved virus. Interestingly, only genes up-regulated by the ancestral virus and unaffected by the evolved virus were significantly enriched in categories related to plant responses to different abiotic and biotic stresses, including systemic acquired resistance and activation of the innate immune resistance.

At face value, the above results support the hypothesis that by adapting to a host, viruses should change and improve the way they interact with the components of the host cell transcriptional network. Therefore, studies of virus-plant interactions should concentrate on naturally coevolved pairs rather than *ad hoc* pairs. While keeping in mind this concern, global profiling experiments will allow identifying sets of genes that are essential for the replication of a given virus, but also other sets that may be required for closely relative viruses and even for unrelated viruses. Furthermore, if all these genes are placed into the context a host regulatory networks, we may identify pathways, rather than individual genes, that may be targets of intervention for therapeutics without undesired side effects.

WHOLE VIRUS GENOME ANALYSIS: CASE STUDY OF CTV

Repeated infections of perennial hosts often result in mixed infections by multiple strains of the same virus or related viruses (7, 92, 110, 131, 143, 144). This occurs, to a lesser extent, in annual crops as well (31, 98, 112). Natural viral complexes create an environment that is conducive to high frequencies of recombination, and consequently appearance of an enormous collection of genetic variants that have potentials to evolve into novel genotypes or strains. In addition, functional complementation of viral proteins in such an environment may nurture mutations that could have been negatively selected in single infections. Studying such complex systems may require novel experimental approaches. An example of these approaches is the analyses of whole genome CTV complexes by high throughput genomic sequencing of viral populations (144, 150). These analyses have revealed detailed, genome-wide information on virus recombination, mutation, and evolution.

CTV, one of the largest plant viruses, is encoded by a positive sense single-stranded RNA molecule of 19.2 to 19.3 kb. Its transmission is through vegetative grafting and by aphids in a semi-persistent manner. Its natural host, citrus, is propagated by budwood grafting and has longevity of more than one hundred years. Repeated transmissions by aphids and vegetative propagation have resulted in an increase in the complexity of CTV populations over hundreds of years. The initial infection may have originated from a single strain, but subsequent infections by different strains occurred during the long history of human cultivation of citrus species, resulting in co-existence of multiple strains (genotypes) in a single host. Similar scenarios of viral accumulation and consequently the existence of viral complexes with multiple strains are common in other viruses that persistently infect perennial, long living trees (80). Within CTV complexes, promiscuous recombination between genomes occurred at remarkably high rates (143, 144). Recent data also suggests extraordinary stability and low mutation rates of CTV (Weng, Z., Dawson, W. O., and Xiong, Z., unpublished). These lead to hypothesis that the high

level of promiscuous RNA recombination compensates for the extreme genome stability and low mutation rate of CTV, and functions as a major force driving the production of genetic variants important for adaptation and evolution. These variants can be selected upon in a new environment and can potentially evolve to become an emerging viral strain. This hypothesis perhaps explains the origin of the sequenced CTV SY568 genome (152), which consists of mosaic sequences from a severe and mild strains (143).

To study these complexes, a high density CTV re-sequencing microarray was designed to simultaneously re-sequence multiple genotypes in CTV populations with high accuracy (Fig. 3) (144, 150). A large number of natural isolates and single-aphid transmitted isolates have been analyzed using this array. Nearly all of the isolates, even some single-aphid-transmitted isolates, were found to contain more than one strain (144). The re-sequencing microarray provided direct visual identification of multiple components in a mixed infection and at the same time re-sequenced the predominant viral sequences in the complex.

This whole genome strategy further showed that CTV complexes comprised one or more predominant genotypes, with one or more genotypes as minor components. For example, a severe stem-pitting isolate from Florida, FL278, contains a predominant T30-like strain. T30 is a mild strain that causes little or no symptoms and does not cause significant economic damage (9), which does not agree with the severity of the disease observed in the source plant. Further analysis using the CTV re-sequencing microarray and real-time PCR revealed a minor genotype (<1% of the population) that resembles a type T36 strain (Table 2), a quick-decline strain that is commonly associated with rapid death of trees on sour orange rootstock (82). The presence of this unusual CTV strain raises a possibility that the T36-like strain may in fact be a contributor to the observed stem-pitting symptoms. This example illustrates that a minor component in a CTV complex can play a significant role in pathogenesis. A thorough knowledge of the genetic

539 composition within a CTV isolate is therefore critical to understand the interaction among
540 different genotypes in a disease complex and their roles in disease development.

541 Evidence for CTV recombination has been documented before (83, 97, 119, 143). As
542 high as 4% of cloned viral genome fragments have been found to be recombinants between two
543 co-infecting strains (143). However, these studies examined only select regions of the large
544 CTV genome. When the recombination analysis is extended to the entire CTV genome by whole
545 genome sequencing analysis, the scale and the degree of promiscuous recombination between
546 co-infecting strains are even more astonishing. For instance, a natural field isolate, FS2-2,
547 contains a CTV complex harboring three distinct strains that are visually identifiable in the
548 hybridized re-sequencing microarray (144) (Fig. 3). A genome-wide 454 sequencing analysis of
549 FS2-2 revealed that a large number of 454 sequencing reads (5%) were recombinants, despite
550 the fact that these sequences were relatively short, with an average length of 256 nucleotides.
551 The recombination events were throughout the entire genome, with the most active
552 recombination occurring toward the 3' half of the CTV genome. FS2-2 contains at least three co-
553 infecting strains: T30, T36, and VT. Promiscuous recombination occurred among all the
554 identified strains. A deeper analysis of 1 kb genomic fragments, sequenced by the traditional
555 Sanger method, corroborated the high level of recombination activity in the FS2-2 complex. A
556 surprisingly large percentage (17.9%) of the cloned molecules was found to be recombinants
557 between the three constituent strains. Additionally, four recombinants possessed two crossover
558 sites, resulting from either a double-crossover or two independent recombination events (144).
559 Further divergence of some recombinants after recombination was also evident in this study.
560 Thus, this data suggests that promiscuous, intergenic recombination can generate a large
561 amount of genetic variants, which could subsequently diverge and evolve to distinct CTV
562 genotypes.

563 An interesting phenomenon in mixed infections of CTV is the lack of apparent cross
564 protection and interference between multiple strains. Even though the 3' halves of multiple CTV
565 genomes within an infected plant share 90% or higher sequence identity, they replicate
566 independently and do not seem to interfere with each other. The inability of the predominant
567 mild T30-like genotype in the above FL278 example to mitigate the effect of the severe T36-like
568 genotype illustrates this failed cross-protection. Nevertheless, successful cross-protection using
569 mild isolates can be achieved (140). Therefore, it is plausible that only strains with highly similar
570 sequences across the entire genome protect against each other, or that in some cases the
571 powerful, three-component RNA silencing suppression system in CTV (50, 91) somehow
572 circumvents the effect of cross protection. Further genome-wide characterization of CTV
573 complexes and other plant viruses will likely shed light on this important yet puzzling biological
574 phenomenon, and provide guidance on effective implementation of cross protection and gene
575 silencing strategies.

576

577 **INTEGRATION INTO DISEASE MANAGEMENT STRATEGIES**

578 Theoretically, an assembly of measures that altogether reduce the **effective population**
579 **size** (N_e), increase the genetic diversity, and maximize the bottleneck effect could gradually
580 exclude a virus from its host species (i.e., virus exclusion = small N_e + large π + low MOI).
581 Unfortunately, the quantification of each one of these parameters is labor intensive, and the
582 magnitude required to obtain the expected effect have not been empirically determined. N_e is
583 generally lower than the census of the population, N, and requires co-inoculation experiments to
584 be estimated. On the other hand, N can be directly estimated by ELISA or real-time PCR and,
585 for practical purposes, it may follow the same trend than N_e in some virus-plant interactions. A
586 greater technological challenge is to develop a more feasible approach of estimating π than the

587 traditional cloning and sequencing. Technological advances such as next generation
588 sequencing systems (15) may provide part of the solution to this task. Significant advancement
589 has been made estimating bottleneck sizes at both ecological and intra-host levels, but these
590 calculations may still need to include the population proportions that represent the estimated
591 MOI. Plant resistance genes may affect N_e, π, or MOI in the desired way. However, few *R*
592 genes, other than *Rz1* and *Rz2* affecting π in sugar beet, have been quantitatively
593 characterized in relation with some of these parameters to predict resistance durability (21, 49).
594 Antivirals based on nucleotide analogs increase π enough to drive the population into lethal
595 mutagenesis (27), but their economic cost and environmental safety in agriculture still need to
596 be evaluated. More viable antiviral strategies may be those based on natural plant resistance
597 mechanisms such as gene silencing or those involving posttranscriptional enzymatic
598 modifications of virus nucleotides.

599 In addition to the genetic plant restriction of virus cell-to-cell and vascular movements,
600 bottleneck narrowing could be achieved by reducing the number of initial infection events to
601 which a plant or plant population is exposed. Traditionally, it has been assumed that the amount
602 of virus particles inoculated in a susceptible plant is epidemiologically irrelevant (57), but it might
603 not be true for some incompatible virus-plant interactions, mainly those where the frequency of
604 virulent genomes is constrained by intra-population interactions.

605 The host environment influences viral population variability by controlling co-infection
606 dynamics, which determine the potential for complementation and recombination (147).
607 Therefore, the role that alternate host reservoirs may have in the generation of virus variation
608 should be taken in consideration during disease risk assessments.

609 *Trans*-complementation among different versions of the same viral element expressed in
610 a common cellular compartment may improve virus robustness through direct reciprocity (e.g., a

611 defective transporter could benefit from an efficient replicator and vice versa) (136). A practical
612 consequence of modeling virus robustness is that, under mutagenic conditions imposed by the
613 host or external mutagens, some virus populations could be eradicated through lethal
614 mutagenesis whereas others would recover following the generation-selection of RB mutations.
615 Serial passage of wild type BNYVV through strongly resistant *Rz2* sugar beets caused an
616 increase of its genetic heterogeneity to such levels that, in some lineages, the virus infection
617 was gradually eliminated while in others its apparent robustness was improved (1).

618 The relationship between virus fitness and the π_W/π_B ratio describe in Figure 2 suggests
619 that, if a virus have reach the adapted stage without causing significant damage to the crop, it
620 most likely will persist in that condition for a long time. Therefore, engaging in a two-arm race
621 with the virus to eradicate the disease may not be a profitable strategy. In conclusion, plant
622 viruses and viroids have become important experimental systems for studying pathogen
623 evolution, and an increasing amount is known about their mutation, recombination, and
624 evolutionary dynamics. The ways in which populations of viruses interact with one or a number
625 of hosts has begun to be probed using high-throughput techniques, and is revealing how small
626 fractions of an infecting population might be driving symptom severity, how rapid viral adaptation
627 to a novel host leads to massive changes in host response to the virus, and how sometimes the
628 hosts that permit the lowest viral titers harbor the greatest viral population diversity. These
629 insights are helping to explain the field data, which often show very little change in viral
630 sequences despite large changes in symptoms, host range, and disease severity. Future work
631 will continue to expand our basic knowledge of phytopathology and direct applied research into
632 how best to control viral and viroid population diversity, effective population size and limit
633 pathogen spread.

634

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644

645 **LITERATURE CITED**

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Textbox 1. Definitions of important terms

Complementation: The rescue of loss-of-function mutants by functional alleles contained in the population.

Effective population size (N_e): Number of individuals capable of producing viable progeny.

Epistasis: The effect of a mutation in one gene over the expression of another gene or mutation of the same genome.

Genetic Bottleneck: A severe reduction in population size.

Genetic drift: Changes in the genetic structure of a population caused by random sampling of haplotypes moving from generation to generation.

Host range mutation: A mutation that affects the host range of a parasite.

Interference: Competition between beneficial mutations to become fixed in the population.

Lethal mutagenesis: Excessive accumulation of mutations in a population that causes population extinction.

Multiplicity of cellular infection (MOI): Relative number of infectious particles that penetrate a cell.

Mutation frequency: Proportion of mutations in a population remaining after the action of selection.

Mutation rate: Proportion of mutations in a population that accumulate prior to the action of host selection.

Mutation-selection balance: The coupling between mutation rate and selection pressures that define the frequency of mutations in a population.

1082 **Nucleotide diversity (π)**: Average number of nucleotide differences per site between any two
1083 randomly chosen haplotypes from a population.

1084 **Positive selection**: Selection of adaptive mutations.

1085 **Purifying (negative) selection**: Conditions that favor the removal of deleterious mutations from
1086 the population.

1087 **Robustness**: Molecular mechanisms that allow for the accumulation of mutations without
1088 concomitant phenotypic change.

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1091 Table 1. Studies of gene expression global profiling for *A. thaliana* in response to viral infection

Virus genus	Virus species	Reference
Caulimovirus	*Cauliflower mosaic virus* (CaMV)	(64)
Cucumovirus	*Cucumber mosaic virus* (CMV)	(77, 96, 145)
Geminivirus	*Mung bean yellow mosaic virus* (MYMV)	(134)
	Cabbage leaf curl virus (CaLCuV)	(10)
Potexvirus	*Potato virus X* (PVX)	(145)
Potyvirus	*Turnip mosaic virus* (TuMV)	(145, 151)
	Plum pox virus (PPV)	(12)
	Tobacco etch virus (TEV)	(5)
Tobamovirus	*Turnip vein clearing virus* (TVCV)	(145)
	Oilseed rape mosaic virus (ORMV)	(145)
	Tobacco mosaic virus (TMV)	(48, 68)

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1097 Table 2. Amounts (fg)[1] of CTV strains in CTV complexes

Strains	CTV complex	
	FS2-2	FL278
VT-like	155.9 ± 9.9	N/A
T30-like	37.1 ± 4.4	86.28 ± 7.7
T36-like	62.0 ± 12.4	0.43 ± 0.2

[1] The amount in fg of CTV cDNA in 1 µg of tissues calculated using standard curves generated for each genotype (Xiong, Z. and Weng, Z., unpublished data).

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FIGURE CAPTIONS

Fig. 1. Average mutation rates (mutations per base per infected cell, except as noted) of viruses and viroids with RNA and ssDNA genomic architectures. The rates of two plant RNA viruses are shown as green squares: TMV (94) and TEV (135). Mutation rates of one RNA bacteriophage and eight RNA animal viruses are shown as orange squares (Measles virus' mutation rate is given as mutations per base per genomic replication event, and cannot be extrapolated to cell infected (125). Similarly, the per base per genomic replication event mutation rate of CChMVd is shown as the green triangle. Two measured mutation rates of single-stranded DNA bacteriophages are shown as blue squares. Rates are from Sanjuán et al. (123) and references therein.

Fig. 2. Model of the relationships between virus fitness and nucleotide diversity of virus populations at the intra- (π_W) and inter- (π_B) isolate levels along virus host adaptation. The arrow represents the direction of the evolutionary steps followed by a virus adapting to a new host genotype (time scales could be significantly different in each one of the four represented evolutionary phases). The intermediate adapting phase is subdivided into an initial genetically incompatible virus-plant interaction and a subsequent compatible interaction at which host resistance has been genetically defeated. Virus adaptations in the old and new hosts are characterized by the lowest π_B and highest virus fitness.

Fig. 3. Images of hybridized re-sequencing microarrays showing multiple strains in isolate FS2-2. The CTV Affymetrix microarray chips were hybridized with target DNA prepared from full length clones of T30 and T36 strains and from full genomic DNA of FS2-2 amplified by RT-PCR. Warm colors represent higher hybridization intensities and cool colors represent lower hybridization intensities. Locations of CTV genomes tiled on the microarray are indicated to the left.