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

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

3

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21

22

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33 (Footnote in first page)

34

35 Virus Acronyms: *African cassava mosaic virus* (ACMV), *Beet necrotic yellow vein virus*

36 (*BNYVV*), *Brome Mosaic virus* (BMV), *Cabbage leaf curl virus* (CaLCuV), *Cauliflower mosaic*

37 *virus* (CaMV), *Chrysanthemum chlorotic mottle viroid* (CChMVd), *Citrus leaf blotch virus*

38 (*CLBV*), *Citrus tristeza virus* (CTV), *Cucumber mosaic virus* (CMV), *Cucumber vein yellowing*

39 *virus* (CVYV), *East African cassava mosaic virus* (EACMV), *Mung bean yellow mosaic virus*

40 (*MYMV*), *Oilseed rape mosaic virus* (ORMV), *Plum pox virus* (PPV), *Potato virus X* (PVX),

41 *Potato virus Y* (PVY), *Rice yellow mottle virus* (RYMV), *Soil-borne wheat mosaic virus*

42 (*SBWMV*), *Tobacco etch virus* (TEV), *Tobacco mild green mosaic virus* (TMGMV), *Tobacco*

43 *mosaic virus* (TMV), *Turnip mosaic virus* (TuMV), *Turnip vein clearing virus* (TVCV), *Wheat*

44 *streak mosaic virus* (WSMV).

45

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

48

49

**ABSTRACT**

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

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66

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68 plant virus evolution: Translating evolutionary insights into better disease management.  
69 Phytopathology (accepted).

70

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

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

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

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

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

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



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

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

170  $2 \times 10^{-5}$  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

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

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

209 The mutation-selection balance is one, but not the only factor determining whether a  
210 mutation will be part of a virus population. **Complementation** between co-infecting viruses can  
211 result in viral genomes carrying deleterious, even lethal, mutations to be maintained in  
212 populations (60). Plant viruses can even develop defective interfering genomes that require at  
213 least one essential component or function be supplied in *trans* by another virus genome (90).  
214 *Viral proteins that generally are shared among particles are involved in coating, cell-to-cell*  
215 *movement, and suppression of gene silencing, but many other proteins may operate in trans*  
216 *exclusively in some virus species.* Defective interfering genomes are distinct from satellite  
217 viruses, which rely on complementation but do not descend from the helper virus. Many  
218 satellites attenuate the effects of the primary virus infection (128), but a minority can increase  
219 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

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

251

252

#### EPIDEMIOLOGICAL DYNAMICS OF VIRUS POPULATIONS

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

258 ***Dynamics of the INTRA-host genetic structure of plant virus***  
259 ***populations.*** Theoretically, the structure of an active virus population changes within a host  
260 individual during the course of a systemic infection. Despite the random emergence of  
261 spontaneous mutations, these structural changes could exhibit deterministic or stochastic  
262 behavior (118). The former has been observed primarily in compatible virus-plant interactions  
263 (i.e., between virulent pathogen and susceptible host), whereas stochastic population structures  
264 are more frequently generated in incompatible interactions (i.e., avirulent pathogen and resistant  
265 host) or during viral host adaptation. Intra-host populations of TMGMV, despite their  
266 heterogeneous composition, exhibit high genetic stability in field infections of *Nicotiana glauca*  
267 (55, 103, 115). High genetic stability also appears to be the norm in several other plant viruses  
268 infecting their compatible hosts (reviewed in by 63). This relative genetic stability suggests that

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

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

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

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

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

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

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



344 within (i.e.,  $\pi_W$ ) 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  $\pi_W$   
346 is higher than at optimal fitness, then, it is expected that the  $\pi_W/\pi_B$  ratio of virus populations will  
347 be related to virus fitness as described in Figure 2. In this way, the ratio  $\pi_W/\pi_B$  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  $\pi_W/\pi_B$  ratio. It was 7.8 to 12.2 times larger in asymptomatic than symptomatic resistant  
355 plants (i.e.,  $\pi_W/\pi_B = 6.1$  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.

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

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



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

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

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

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

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

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

465 To test whether adaptation to a host may result in changes in mRNA profile, Agudelo-  
466 Romero *et al.* (6) performed an evolution experiment adapting TEV to the susceptible ecotype  
467 *Ler-0* of *A. thaliana* (25). The TEV clone used as the ancestral virus was able of systemically  
468 infect *Ler-0* plants although the infection was asymptomatic. After 17 serial passages, the  
469 resulting virus (labeled as TEV-At17) accumulated three orders of magnitude more than the  
470 ancestral one per gram of infected tissue, its infectivity was 100% (compared with the low 10%  
471 of the ancestral genotype) and induced severe symptoms including stunting, etching, and leaf  
472 malformation. A single amino acid substitution in the VPg was enough to trigger these  
473 symptoms. TEV-At17 infection caused the differential expression of a total of 505 up-regulated  
474 and 1335 down-regulated plant genes relative to its ancestor virus (Figure 3 in 6). Both viruses  
475 also differentially affected the expression of transcription factors, with 51 up-regulated and 84  
476 down-regulated only by the evolved virus. Interestingly, only genes up-regulated by the  
477 ancestral virus and unaffected by the evolved virus were significantly enriched in categories  
478 related to plant responses to different abiotic and biotic stresses, including systemic acquired  
479 resistance and activation of the innate immune resistance.

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

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**WHOLE VIRUS GENOME ANALYSIS: CASE STUDY OF CTV**

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

502 CTV, one of the largest plant viruses, is encoded by a positive sense single-stranded  
503 RNA molecule of 19.2 to 19.3 kb. Its transmission is through vegetative grafting and by aphids  
504 in a semi-persistent manner. Its natural host, citrus, is propagated by budwood grafting and has  
505 longevity of more than one hundred years. Repeated transmissions by aphids and vegetative  
506 propagation have resulted in an increase in the complexity of CTV populations over hundreds of  
507 years. The initial infection may have originated from a single strain, but subsequent infections by  
508 different strains occurred during the long history of human cultivation of citrus species, resulting  
509 in co-existence of multiple strains (genotypes) in a single host. Similar scenarios of viral  
510 accumulation and consequently the existence of viral complexes with multiple strains are  
511 common in other viruses that persistently infect perennial, long living trees (80). Within CTV  
512 complexes, promiscuous recombination between genomes occurred at remarkably high rates  
513 (143, 144). Recent data also suggests extraordinary stability and low mutation rates of CTV  
514 (Weng, Z., Dawson, W. O., and Xiong, Z., unpublished). These lead to hypothesis that the high

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

521 To study these complexes, a high density CTV re-sequencing microarray was designed  
522 to simultaneously re-sequence multiple genotypes in CTV populations with high accuracy (Fig.  
523 3) (144, 150). A large number of natural isolates and single-aphid transmitted isolates have  
524 been analyzed using this array. Nearly all of the isolates, even some single-aphid-transmitted  
525 isolates, were found to contain more than one strain (144). The re-sequencing microarray  
526 provided direct visual identification of multiple components in a mixed infection and at the same  
527 time re-sequenced the predominant viral sequences in the complex.

528 This whole genome strategy further showed that CTV complexes comprised one or more  
529 predominant genotypes, with one or more genotypes as minor components. For example, a  
530 severe stem-pitting isolate from Florida, FL278, contains a predominant T30-like strain. T30 is a  
531 mild strain that causes little or no symptoms and does not cause significant economic damage  
532 (9), which does not agree with the severity of the disease observed in the source plant. Further  
533 analysis using the CTV re-sequencing microarray and real-time PCR revealed a minor genotype  
534 (<1% of the population) that resembles a type T36 strain (Table 2), a quick-decline strain that is  
535 commonly associated with rapid death of trees on sour orange rootstock (82). The presence of  
536 this unusual CTV strain raises a possibility that the T36-like strain may in fact be a contributor to  
537 the observed stem-pitting symptoms. This example illustrates that a minor component in a CTV  
538 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.

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#### 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  $\pi$  + 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  $\pi$  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  $N_e$ ,  $\pi$ , or MOI in the desired way. However, few  $R$   
592 genes, other than  $Rz1$  and  $Rz2$  affecting  $\pi$  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  $\pi$  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  $\pi_W/\pi_B$  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.

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1061 Textbox 1. Definitions of important terms

1062 **Complementation:** The rescue of loss-of-function mutants by functional alleles contained in the  
1063 population.

1064 **Effective population size ( $N_e$ ):** Number of individuals capable of producing viable progeny.

1065 **Epistasis:** The effect of a mutation in one gene over the expression of another gene or mutation  
1066 of the same genome.

1067 **Genetic Bottleneck:** A severe reduction in population size.

1068 **Genetic drift:** Changes in the genetic structure of a population caused by random sampling of  
1069 haplotypes moving from generation to generation.

1070 **Host range mutation:** A mutation that affects the host range of a parasite.

1071 **Interference:** Competition between beneficial mutations to become fixed in the population.

1072 **Lethal mutagenesis:** Excessive accumulation of mutations in a population that causes  
1073 population extinction.

1074 **Multiplicity of cellular infection (MOI):** Relative number of infectious particles that penetrate a  
1075 cell.

1076 **Mutation frequency:** Proportion of mutations in a population remaining after the action of  
1077 selection.

1078 **Mutation rate:** Proportion of mutations in a population that accumulate prior to the action of  
1079 host selection.

1080 **Mutation-selection balance:** The coupling between mutation rate and selection pressures that  
1081 define the frequency of mutations in a population.

1082 **Nucleotide diversity ( $\pi$ ):** 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	<i>Cauliflower mosaic virus</i> (CaMV)	(64)
Cucumovirus	<i>Cucumber mosaic virus</i> (CMV)	(77, 96, 145)
Geminivirus	<i>Mung bean yellow mosaic virus</i> (MYMV)	(134)
	<i>Cabbage leaf curl virus</i> (CaLCuV)	(10)
Potexvirus	<i>Potato virus X</i> (PVX)	(145)
Potyvirus	<i>Turnip mosaic virus</i> (TuMV)	(145, 151)
	<i>Plum pox virus</i> (PPV)	(12)
	<i>Tobacco etch virus</i> (TEV)	(5)
Tobamovirus	<i>Turnip vein clearing virus</i> (TVCV)	(145)
	<i>Oilseed rape mosaic virus</i> (ORMV)	(145)
	<i>Tobacco mosaic virus</i> (TMV)	(48, 68)

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Table 2. Amounts (fg)<sup>1</sup> 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

<sup>1</sup> 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**

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1102 **Fig. 1.** Average mutation rates (mutations per base per infected cell, except as noted) of viruses  
1103 and viroids with RNA and ssDNA genomic architectures. The rates of two plant RNA viruses are  
1104 shown as green squares: TMV (94) and TEV (135). Mutation rates of one RNA bacteriophage  
1105 and eight RNA animal viruses are shown as orange squares (Measles virus' mutation rate is  
1106 given as mutations per base per genomic replication event, and cannot be extrapolated to cell  
1107 infected (125). Similarly, the per base per genomic replication event mutation rate of CChMVd is  
1108 shown as the green triangle. Two measured mutation rates of single-stranded DNA  
1109 bacteriophages are shown as blue squares. Rates are from Sanjuán et al. (123) and references  
1110 therein.

1111 **Fig. 2.** Model of the relationships between virus fitness and nucleotide diversity of virus  
1112 populations at the intra- ( $\pi_W$ ) and inter- ( $\pi_B$ ) isolate levels along virus host adaptation. The arrow  
1113 represents the direction of the evolutionary steps followed by a virus adapting to a new host  
1114 genotype (time scales could be significantly different in each one of the four represented  
1115 evolutionary phases). The intermediate adapting phase is subdivided into an initial genetically  
1116 incompatible virus-plant interaction and a subsequent compatible interaction at which host  
1117 resistance has been genetically defeated. Virus adaptations in the old and new hosts are  
1118 characterized by the lowest  $\pi_B$  and highest virus fitness.

1119 **Fig. 3.** Images of hybridized re-sequencing microarrays showing multiple strains in isolate FS2-  
1120 2. The CTV Affymetrix microarray chips were hybridized with target DNA prepared from full  
1121 length clones of T30 and T36 strains and from full genomic DNA of FS2-2 amplified by RT-PCR.  
1122 Warm colors represent higher hybridization intensities and cool colors represent lower  
1123 hybridization intensities. Locations of CTV genomes tiled on the microarray are indicated to the  
1124 left.