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REVIEW ARTICLE

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Variation and evolution of plant virus populations

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Abstract Over the last 15 years, interest in plant virus evolution has re-emerged, as shown by the increasing number of papers published on this subject. In recent times, research in plant virus evolution has been viewed from a molecular, rather than populational, standpoint, and there is a need for work aimed at understanding the processes involved in plant virus evolution. However, accumulated data from analyses of experimental and natural populations of plant viruses are beginning to delineate some trends that often run contrary to accepted opinion: (1) high mutation rates are not necessarily adaptive, as a large fraction of the mutations are deleterious or lethal; (2) in spite of high potential for genetic variation, populations of plant viruses are not highly variable, and genetic stability is the rule rather than the exception; (3) the degree of constriction of genetic variation in virus-encoded proteins is similar to that in their eukaryotic hosts and vectors; and (4) in spite of huge census sizes of plant virus populations, selection is not the sole factor that shapes their evolution, and genetic drift may be important. Here, we review recent advances in understanding plant virus evolution, and describe the experimental and analytical methods most suited to this purpose.

Keywords Selection · Genetic drift · Mutation · Population genetics · Recombination

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Introduction

Populations of plant viruses are genetically heterogeneous. As is the case with all living entities, reproduction may result in the generation of individuals that differ genetically from their parents; these are called mutants or, more vaguely, variants. The frequency distribution of genetic variants in the population of an organism (i.e. the genetic structure of the population) may change with time, and this process is called evolution.

The analysis of the genetic structure and evolution of populations is a crucial area of biology. In the case of pathogens, which include plant viruses, a knowledge of their evolution is crucial to the development of efficient and stable control strategies, as these often fail due to evolution of the pathogen population; overcoming of resistance genes by resistance-breaking pathotypes is but the most noticeable instance. The advent of analytical techniques, such as those allowing the fast determination of nucleotide sequences, and the need to analyse the possibilities and risks of new control strategies, such as the use of virus-resistant transgenic plants, has resulted in renewed interest in the study of the genetic variability and evolution of plant viruses. This has lead to a recent increase in the number of publications dealing with the variability and evolution of plant viruses, and in reviews on this and related subjects (e.g. [33, 34, 69]). A large part of the recent work reflects the molecular, rather than populational, orientation of their authors. Hence, in this review we have stressed the experimental and analytical approaches most suited for the analysis of the genetic variation of virus populations.

Sources of genetic variation

Genetic variation is generated by errors occurring during the replication of genomes. For viruses, the two main types of error so far described are mutation (sensu stricto) and recombination. Mutation is the process that results in differences between the nucleotides incorporated into the

daughter strand during nucleic acid replication and those in the template. Mutation is the initial source of variation in populations, hence the interest in estimating the rate at which it occurs [17]. Let us differentiate here between the mutation rate and the observed mutant frequency in the analysed population. These two variables may differ broadly, as an unknown fraction of the generated mutants is deleterious and will be eliminated from the population by selection. The relationship between mutation frequency and mutation rate may also be different according to the replication strategy and life history of the virus [17]. Estimates of mutation rate reported for lytic RNA viruses infecting mammals are in the range of 10^{-4}–10^{-5} misincorporations per nucleotide per replication round, which results in about one error (0.76, 96% confidence interval 0.18–1.07) per genome per replication cycle [16]. A recent estimate for the plant RNA virus *Tobacco mosaic virus* (TMV), based on the detection of mutants lethal for cell-to-cell movement, gave an estimate of the mutation rate per genome of 0.10–0.13, which is of the same order of magnitude as estimates for the lytic RNA viruses [52]. The same study also analysed the nature of these mutations, and was the first report on the mutational spectrum of an RNA virus. A large fraction (69%) of the mutations were insertions and deletions, and half of them involved from three to many bases. Such a ratio of base substitutions to insertions and deletions had only been reported previously for a retrovirus and for an archeon [35, 63]. Another outstanding trait is that a large fraction of the mutants (35%) were multiple mutants, a characteristic not shared with any other reported mutational spectrum. These data show that most mutations in TMV, and possibly in other RNA viruses, are not of an adaptive nature, and support the view that the high mutation rates of RNA viruses is due to the need for rapid replication of their chemically unstable RNA genome rather than being an evolutionary strategy [16]. The mutation rate for the dsRNA phage $\phi 6$ is similar to that of TMV [12], and this might also be so for plant viruses with dsRNA genomes. Mutation rates are an order of magnitude smaller for retroviruses than for RNA viruses [17]. These values might be extrapolated to plant DNA viruses that replicate by reverse transcription of an RNA intermediate. For viruses with large dsDNA genomes, mutation rates per genome are about 0.003 per replication round [17]. It is not known if these values can be applied to the small ssDNA plant viruses, for which no estimates of mutation rate are available.

Recombination is the process by which segments of genetic information are switched between the nucleotide strands of different genetic variants during the process of replication. Thus, recombination results in genetic exchange. Sequence analyses of populations of various RNA and DNA plant viruses provide evidence that recombination may be a major source of evolutionary variation, and it might be particularly important for certain virus groups (cf. [33]). At the population level, recombination may result in dramatic changes in the biological properties of the virus, with major epidemiological consequences, including the appearance of resistance-breaking strains or the acquisition of broader host ranges [49, 56]. Concerns about gene flow from transgenic plants with pathogen-derived resistances to viruses and on to populations of infecting viruses have resulted in new efforts to analyse the mechanisms of recombination and their role in virus evolution [72]. Nonetheless, if estimates of mutation rates are scant, even less information is available on recombination frequencies in the absence of selection. Recombination frequencies in RNA viruses—which depend on the degree of sequence similarity between the sequences involved, the distance between the markers used to identify the recombinants, and the presence of recombination hot spots—can be as high as mutation rates (see [33]).

Genetic exchange may also result from re-assortment of genomic segments in viruses with a segmented genome, a process also called pseudorecombination by plant virologists. There is evidence that re-assortment occurs in natural populations of plant viruses [25], and it may play a major role in virus evolution (e.g. [75]). As for recombination, the effects of reassortment on virus biology may be dramatic, but its role in the genetic structure of virus populations has not been much analysed. Most evidence is for selection against reassortants, and for co-adaptation of genomic segments [33].

Association with new nucleic acid molecules may alter virus pathogenicity and be another source of genetic variation. This may result from associations between viruses, and between viruses and satellites, which are common in plant viruses. Examples include the presence of satellite RNAs (satRNAs) of cucumber mosaic virus (CMV) resulting in strains that cause systemic necrosis in tomato, or the role of groundnut rosette virus satRNA on the pathogenicity and epidemiology of groundnut rosette disease [31, 60].

Analysis and evidence for genetic variation of plant viruses

Different approaches may be used to analyse the genetic variation of plant viruses. Initially, variants were characterised by differences in biological properties such as the symptoms they caused in different host plant species, their host range, or vector transmission properties. The development of techniques that allowed the characterisation of properties of the virus other than those related to its interaction with the hosts and vectors resulted in a dramatic change of perspective in these studies. These techniques are often more sensitive and reproducible, and allow the typification of more isolates than bioassays. Moreover, these techniques allow the typification of characters that could be neutral (i.e. not subject to selection); hence, they would be appropriate to analyse the genetic structure of virus populations. First to be used were techniques that allowed the characterisation of viral structural protein(s), such as the electrophoretic mobility of virions and coat protein(s) (CP)

subunits, peptide mapping, amino acid composition and sequence analysis of the CP, and its immunology, including the use of monoclonal antibodies and epitope mapping. Later, molecular techniques allowing the analysis of the virus genome became available, and these are most favoured at present.

The choice of a given analytical technique should depend on the goal of the analysis, as well as on the sensitivity and cost of the technique. However, there is a difference between techniques that provide only qualitative data, i.e. that which can be used to identify variants, and those that provide information that can be used to quantify how different the identified variants are, i.e. to estimate their genetic distance. Estimates of genetic distances can derive from data on the amino acid composition of the viral proteins, or from serological comparisons using both polyclonal and monoclonal antibodies [73]. Genetic distances can also be estimated from ribonuclease T1 fingerprint [59] and restriction fragment length polymorphism (RFLP) analyses [62]. Two procedures often used by plant virologists, ribonuclease protection assay of a labeled cRNA probe (RPA) and single-stranded conformation polymorphisms (SSCP), yield results that depend on sequence context and do not allow the direct estimation of genetic distances [6, 44]. Of course, analyses of the nucleotide sequences of viral genes provide the most detailed data both to identify genetic variants and to estimate the genetic distance between them.

The initial evidence for genetic variation of plant viruses was the observation that isolates causing different symptoms could derive from the same virus source. This dates back to the 1920s and 1930s [46]. (For a discussion of the terms ''isolate'', ''strain'', ''mutant'' and ''variant'' see [33]). It was soon observed that serial passages in different host plant species also resulted in a change of virus traits that was called host adaptation [76]. Host adaptation was interpreted as the selection of variants present in the original virus population or newly generated from it. In this way the heterogeneous nature of virus populations, including the laboratory stocks used in these experiments, was recognised at an early date. Molecular analyses showed that biological cloning by single-lesion passages did not eliminate heterogeneity in TMV, as new variants could arise by mutation [32]. The heterogeneity of RNA virus populations was further shown by the analysis of populations obtained by the multiplication of inocula derived from biologically active cDNA clones (e.g. [1, 4, 48]). Thus, both early and recent work has shown that mutants evolve through virus multiplication from biologically or molecularly cloned inocula, and that a laboratory stock of a plant virus isolate is always a heterogeneous population comprising different variants. The structure of this population, first described for *Tobacco mild green mosaic virus* (TMGMV) [68], usually consists of a major genotype plus a set of minor variants newly generated by mutation, or kept at low frequency by selection. This genetic structure, which had been reported previously for bacterial and animal RNA viruses, was named a quasispecies and had been associated with the high error rates of RNA-dependent RNA polymerases (RdRp). The quasispecies theory has recently been abundantly reviewed (see [14]) and discussed [13, 42]. Note that neither the genetic structure of populations of viroids (e.g. [74]), nor that of plant DNA viruses (e.g. [2]) seem to differ qualitatively from that of RNA viruses. Plant virologists should be aware of the full meaning of the term quasispecies, which should not be applied to describe just any heterogeneous set of sequences, as is often the case.

Evolutionary factors in virus populations

Two major evolutionary processes determine the frequency distribution in the virus population of the genetic variants generated by mutation or genetic exchange: genetic drift, and selection. Because populations may not be large enough to ensure that each variant will be equally represented in the next generation, random effects would occur during the transmission of genetic traits to new generations; this random process is called genetic drift. Selection is a directional process by which the frequencies of variants that are the fittest in a given environment will increase in the population (positive selection) whereas those of less fit variants will decrease (negative or purifying selection). A consequence of selection is that, in a population large enough (ideally of infinite size) for all variants to have progeny in the next generation, the frequency at equilibrium of a variant provides an estimate of its fitness.

Genetic drift

Populations of plant viruses can reach very large sizes within one infected plant. For TMV, for instance, the number of particles in an infected tobacco leaf has been estimated to be in the range of 10^{11}–10^{12} [37, 52]. This census population size might differ by a large factor from the effective population size, which is the number of individuals that contribute equally to the following generation. The effective population size, and not the census population size, is what matters for the evolution of the virus population, a point often overlooked in the virological literature. In a population of an RNA virus such as TMV, the effective population size may be much smaller than the actual population size, because a large fraction of the population will consist of mutants that will not multiply, as suggested by their low intrinsic infectivity (in the range 10^{3}–10^{4}; [32]). In addition, infection of a new host may be started by a very small number of virus particles (one, in theory), which will reduce even more the effective population size. Estimates, under different assumptions, of the effective population size of *Human immunodeficiency virus* type I in an infected patient indicate that it would be several

orders of magnitude smaller than the census population [29]. Our work with TMV shows that founder numbers in the colonisation of a new leaf are small, indicating that severe bottlenecks occur during systemic colonisation of tobacco plants, and that effective numbers could be much smaller than census numbers [70]. Thus, genetic drift may be important in the evolution of plant virus populations, as shown by the few detailed analyses reported on changes in population structure during the colonisation of new organs or new host plants [8, 36, 70].

An important issue regarding the effect of population bottlenecks in the evolution of viruses is that they can result in effective population sizes below the threshold needed to ensure the transmission of the fittest genotypes, as shown experimentally with bacterial and animal RNA viruses [11, 18]. As a result, the viral population becomes progressively dominated by less fit genotypes—a process known as Muller's ratchet—and will succumb by a mutational meltdown [51]. Mutational meltdown can occur in nature as a result of the interaction between two different virus populations [26]. The tobamoviruses TMV and TMGMV were found infecting *Nicotiana glauca* plants collected in Australia before 1950, but only TMGMV has been isolated from more recent specimens. It has been shown that in mixed infections in this host, the accumulation of TMGMV is not affected, but that of TMV is severely reduced as compared with single infections. Also, nucleotide sequence comparisons of TMV and TMGMV isolates have shown that the TMGMV populations are large enough to ensure the transmission of the fittest genotype, which does not change with time, whereas mutations accumulate in TMV with time. Thus, a probable cause for the disappearance of TMV from the *N. glauca* population is that a mutational meltdown has occurred as a consequence of co-infection with TMGMV.

Selection

Selection is the process most frequently invoked in the literature to explain virus evolution, but this is not always based on evidence. The effects of selection and genetic drift are often difficult to separate, because selection also results in a decrease in the population diversity and may increase diversity between populations if these are under different selection pressures. Selection can be associated with every factor in the virus life cycle. For instance, selection pressures associated with the maintenance of functional structures have been documented for the CP of tobamoviruses [3], as well as for non-coding regions that play a role in the replication of viral genomes (e.g. [7, 9]). The maintenance of a functional structure would be a primary factor for subviral non-coding RNAs, as has been shown for satellite RNAs and viroids (e.g. [4, 24]).

Another obvious group of selection factors will be those associated with the host plant. In this case, evidence comes from the host-adaptation experiments already mentioned. Consistent selection of different variants in different host plants has been well documented [15, 43, 47]. The differentiation of natural populations according to the host plant can also be taken as evidence of host-associated selection, as reported for both viruses and viroids (e.g. [45, 55, 71]). Perhaps the best evidence of host-associated selection derives from the well known phenomenon of the overcoming of resistance genes. This phenomenon has occasionally been analysed in detail at the population level, a good example being the classical work of Pelham on the overcoming of *Tm-1* resistance in tomato by tomato mosaic virus (ToMV) [64]. Overcoming of resistance has been analysed recently [30, 39]. Virulence, defined as the effect of a pathogen in decreasing the fitness of its host (rather than relative to the replicating ability of the virus), is a major feature of pathogens that may be selected for and which might play an important role in virus evolution. Virulence being a key attribute of pathogens, this is an issue that has received considerable theoretical attention [5]. However, few experimental results have been reported. Data that indicate selection of virulence in *Beet curly top virus* (BCTV) and *Rice grassy stunt virus* have been reported [38]. The evolution of virulence in populations of CMV and its satRNA depend on trade-offs between increased virulence and decreased transmissibility; consequently, not only the size, but also the evolution, of the virus population depends on the population dynamics of the aphid vectors [19, 20, 21]. Because of their effect on the fitness of the host plant, viruses can determine the size and/or the genetic composition of plant populations, which in turn could affect virus evolution. In fact, it has been shown that virus infection may affect the fitness of wild plants and weeds, as well that of crops [27, 54]. Detailed analyses of the role of virus infection in plant populations are scarce, a good example being a study on wild *Brassica* species in England [66].

A third group of selection factors would be those associated with the interaction of viruses and their vectors. Initial evidence for vector-associated selection was the loss of vector transmissibility after repeated non-vector passaging of viruses, first reported for *Wound tumour virus* [67], and often thereafter [28]. Evidence for vector-associated selection also derives from reports of the selection of particular genomic combinations in viruses with segmented genomes upon vector transmission (e.g. [65]). Geographically-related antigenic variation in begomoviruses may also be evidence of vector selection [40]. Like their host plants, viruses may also affect the genetic structure and dynamics of insect vector populations. In fact, attraction and preference of aphids may differ between virus-infected and healthy plants [23], and virus infection may modify the reproductive potential of aphids [22]. In addition, viruses may have a negative impact on the fitness of their vectors, as might be the case for *Rice stripe virus*, which is propagative in its delphacid vector [41].

Evidence for negative and positive selection on plant virus genes

Sequence analyses show that, in most instances, the selection acting on virus genes is negative. The degree of negative selection in genes, and the degree of functional constraint for the maintenance of the encoded protein sequence can be estimated from the ratio between nucleotide diversities at non-synonymous and synonymous positions (d_{NS}/d_S ratio). As nucleotide diversity is a measure of the probability that the base at a given position differs between two randomly chosen individuals from the population, this ratio indicates the amount of variation in the nucleic acid that results in variation in the encoded protein. Analysis of this ratio for structural and non-structural proteins of a number of RNA and DNA viruses (see Table 1 in [33]) shows that they are similar to those reported for RNA and DNA viruses infecting animals (see Table 7.9 in [50]), and all fall within the range reported for DNA-encoded genes of cellular organisms (Table 5.4 in [61]). Thus, virus-encoded proteins are not less constrained than those of their eukaryotic hosts and vectors, which suggests that the need to establish functional interactions with host- and vector-encoded factors is constraining the variability of virus-encoded proteins. Another major source of constraint could be the well-documented multifunctionality of virus-encoded proteins, which would result in different selective constraints corresponding to various functions, and hence the protein would never be optimised for just one of its functions. For instance, the helper component in potyvirus transmission also plays roles in the proteolytic processing of the genome translation product, in systemic movement, and in countering the silencing reaction of the host plant [10]. The fact that the function of the helper component in aphid transmission is easily lost upon mechanical passage (see above) suggests that trade-offs occur for the optimisation of its different functions. Constraints to the genetic variation of plant virus population are also shown by the higher durability of plant resistance to viruses, as compared to phytopathogenic fungi or bacteria. It is often the case that resistance-breaking genotypes do not become prevalent in the virus population, indicating that the genetic changes that result in resistance breaking incur a cost [30].

Even if the analysis of complete virus genes shows that negative selection is operating, positive selection may be acting in particular domains of the viral proteins, and be evidenced by more detailed analyses of the encoding sequence, as reported for *Potato virus Y* [58]. Positive selection clearly acts with resistance-breaking isolates, as shown for ToMV isolates that overcome the resistance of the *Tm-1* gene [64].

Complementation of deleterious variants may counter the effects of selection

The effects of selection on deleterious mutants may be countered by complementation in multiply infected cells if the function affected by the mutation is provided in trans by fully competent genotypes. This should be of particular importance for RNA viruses, which will generate many mutants that share the cell environment with the competent parental variant. Complementation could result, for instance, in the maintenance of more virulent, less fit variants, which could have important consequences for pathology at large. Complementation of replication, movement and transmission has been often described in experimental systems, but its role in virus evolution has been overlooked, and few attempts have been made to quantify its effects. The analysis of a *Tomato aspermy virus* mutant lethal for cell-to-cell movement that replicated more efficiently than the wild type provided a lower threshold estimate of probability (0.13) that the non-functional individual will move cell-to-cell as compared to the functional one. This resulted in an equilibrium frequency of 0.76 for the movement mutant, indicating the importance of complementation in shaping the genetic structure of the population [57]. Mansky et al. [53] have shown that in soybean plants carrying the resistance gene *Rsv*, previous infection by a virulent strain of *Soybean mosaic virus* complemented infection by a second strain to which these plants were otherwise immune. Their data allow a lower limit on the efficiency of complementation of 0.20 to be set, and illustrate the potential importance of this largely unexplored phenomenon in virus evolution in the field.

Diversity of plant virus populations

The high potential to vary need not result in fast rates of change in virus populations, and sequence data accumulated over the last 10 years show that genetic stability is the rule, rather than the exception, in natural populations of plant viruses. This perception is often blurred by the analytical methods used to compare isolates of a virus population. Population diversity can be defined as the probability that two randomly chosen isolates from a population are different. More precise estimates of population diversity are obtained if there is information on how different those randomly chosen isolates are, i.e. what is the genetic distance between them (see chapter 10 in [61]). Thus, population diversity depends on three parameters: number of haplotypes present in the population, frequency with which each haplotype is present in the population, and genetic distances between the existing haplotypes. Most work on plant virus populations analyse only the number of haplotypes present in the population. Since this number is usually large relative to the number of isolates analysed, authors often conclude that the analysed population is highly variable, which perhaps is not the case. In most instances, published work does not include information about the frequencies in the population of each identified haplotype. It is necessary to stress that the number of haplotypes identified, and the frequency of the more prevalent one, depend on the size of the genomic target analysed,

on the sample size, and on the analytical method, as discussed in a previous section. Researchers should keep in mind that the size of the sample needed to estimate the diversity of a population, or to compare it with others, depends on the population diversity (i.e. on the variance of the analysed trait). It is often considered that a high number of isolates needs to be analysed, which might not increase significantly the precision of the estimate of the population diversity and establishes a dangerous trend among researchers and reviewers.

Analyses of population diversity from the data available for some virus species indicate in all instances a low genetic diversity, i.e. below 0.10 (see Table 2 in [33]). No correlation was found between population diversity and any trait in the virus life cycle, such as mode of transmission, type of host plant, or nature (DNA or RNA) of the virus genome. The current opinion that RNA viruses are very variable derives mostly from the analysis of viruses such as *Influenza A virus, Hepatitis C virus* and *Foot and mouth disease virus,* and these may be exceptions rather than the rule, even for animal viruses. No highly variable viruses have yet been reported in plants.

The genetic stability of plant virus populations is further illustrated by the high numbers of host-pathogen systems in which resistant-breaking pathotypes have been reported, but which do not become established in the virus population and the resistance factor remains effective for long periods of time [30]. For some viruses (e.g. BCTV, *Raspberry ringspot virus,* ToMV, *Turnip mosaic virus*), it has been shown that resistance-breaking strains are less fit because of poor transmissibility or poor competitive ability in the crop or in other host plants [30], i.e. increased virulence has a cost. The multiple constraints to the evolution of viral proteins discussed above may be an important factor determining the stability of plant virus populations.

Final comments

Significant work on the variability and structure of plant virus populations was published prior to the advent of molecular virology. Over the last 15 years molecular analyses of plant virus variation have been increasingly reported. However, studies aimed at quantifying the variability of virus populations, or characterisation of their genetic structure, are comparatively scarce. In spite of this, some trends emerge from the analysis of the accumulated data that may be contrary to preconceived ideas on plant virus populations. A major one is that, without exception, analysed populations of plant viruses are genetically stable, and this is so regardless of the many of haplotypes that may occur in the population. More than 20 years ago Harrison [38] already stressed that resistance genes in crops were less often overcome by viruses than by other plant pathogens; he suggested that virus populations are smaller and genetically more stable than those of other plant pathogens. Recent work and analyses have largely confirmed his predictions. Analyses of the diversity of virus genes have also shown that they are not more variable than those of their eukaryotic hosts and vectors. Evidence has also accumulated on the role of some of the factors that may model the evolution of plant viruses. Nevertheless, in most cases it is not possible to relate these factors to the genetic structure of the few virus populations so far analysed. Obviously, more effort is required to understand the evolution of plant viruses.

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