Annual Review of Virology

Natural Selection, Intracellular Bottlenecks of Virus Populations, and Viral Superinfection Exclusion

Camila Perdoncini Carvalho, Ruifan Ren, Junping Han, and Feng Qu

Department of Plant Pathology, The Ohio State University, Wooster, Ohio, USA; email: qu.28@osu.edu

Annu. Rev. Virol. 2022. 9:4.1–4.17

The *Annual Review of Virology* is online at virology.annualreviews.org

https://doi.org/10.1146/annurev-virology-100520-114758

Keywords

natural selection, plus-strand RNA virus, population bottleneck, superinfection exclusion, mutation, replication

Abstract

Natural selection acts on cellular organisms by ensuring the genes responsible for an advantageous phenotype consistently reap the phenotypic advantage. This is possible because reproductive cells of these organisms are almost always haploid, separating the beneficial gene from its rival allele at every generation. How natural selection acts on plus-strand RNA viruses is unclear because these viruses frequently load host cells with numerous genome copies and replicate thousands of progeny genomes in each cell. Recent studies suggest that these viruses encode the Bottleneck, Isolate, Amplify, Select (BIAS) mechanism that blocks all but a few viral genome copies from replication, thus creating the environment in which the bottleneck-escaping viral genome copies are isolated from each other, allowing natural selection to reward beneficial mutations and purge lethal errors. This BIAS mechanism also blocks the genomes of highly homologous superinfecting viruses, thus explaining cellular-level superinfection exclusion.

1. INTRODUCTION

An informed discussion on how natural selection acts on viruses demands a concise definition of natural selection. At its core, natural selection consists of two interrelated rules. First, selection acts on phenotypic differences. This simply means that, within a population of the same species living under nearly identical conditions, some individuals may manifest certain physical or behavioral characteristics, namely phenotypes, that enable them to outcompete other individuals. Individuals expressing such advantageous phenotypes hence become more numerous in the population over time (1). This is best illustrated by the body color evolution of peppered moths in areas of England during a time of worsening industrial pollution. Peppered moths with black bodies were rare before 1848, yet their numbers increased steadily during the next 50 years, eventually accounting for 98% of all peppered moths in the affected areas by 1895. It was later demonstrated that in an environment heavily contaminated with soot, the black-bodied moths had the phenotypic advantage of blending in with their surroundings and thus surviving from predatory birds in larger numbers (2). In this example, the black body color was a phenotype favored by natural selection.

The second rule of natural selection is that the phenotypic differences under selection must be heritable, meaning they must be caused by differences in specific genes, frequently in the form of allelic variants of the same ancestral gene. Only then can the selected-for phenotype accrue the phenotypic benefits to the very individual carrying the corresponding gene, making this gene more likely to survive and reproduce than its rival alleles. The increased frequency of the beneficial gene in the population in turn enables more individuals to express the advantageous phenotype, perpetuating the virtuous cycle of natural selection.

From this second rule derives another condition that is often overlooked but is nevertheless essential for natural selection to occur: The beneficial gene/allele must be the primary beneficiary of the advantageous phenotype. This condition is rarely unmet for cellular organisms simply because most cellular organisms are either haploid or diploid, whose cells contain no more than two sets of homologous chromosomes. As a result, a phenotypic advantage is relayed to no more than two types of germline cells, with at least one half of these germline cells harboring the beneficial gene. While the advantage is also conferred to the gene allelic to the beneficial gene in a sexually reproducing heterozygote, such a benefit is transitory because the rival allele is soon separated from the beneficial allele in the subsequent generation. In short, the gene responsible for the selected-for phenotype must be separated from its rival alleles to allow for the perpetuation of the phenotypic advantage and thus the gene itself.

The separation of gene alleles is even more crucial for purifying selection. To better illustrate this point, let us go back to the example of positive selection among living peppered moth individuals. Peppered moths with nonblack body colors were born alive but were more easily caught and killed by predatory birds; hence, they left behind fewer descendants, causing the underlying genes to decrease in frequency over multiple generations. By contrast, there were peppered moth embryos that never developed into live larvae due to lethal mutations in essential genes. For instance, certain loss-of-function mutations in a DNA polymerase gene arrest cell division at a very early stage of embryo development (3), thereby eliminating such mutations through purifying selection. Purifying selection cannot occur unless the lethal mutation is separated from its functional alleles. Indeed, most of the hereditary diseases caused by loss-of-function mutant genes are recessive and manifest their fatal phenotypes only when the mutated genes are homozygous, being completely separated from their functional alleles.

To reiterate, in cellular organisms the separation of gene alleles from each other is guaranteed because their germline cells carry only one set of chromosomes. However, such separation becomes a huge challenge for viruses, as thousands of genome copies of the same virus can coexist

in a single cell. Nevertheless, quite a few virologists seem to believe that many of the coexisting genome copies can simultaneously replicate in the same cell and for multiple cycles (4). How can natural selection act on viruses to facilitate the rapid spread of beneficial (to virus) mutants, such as the Delta variant of severe acute respiratory syndrome coronavirus 2, if the mutant genome has to share the encoded advantage with many rival genomes (5)?

In this review, we try to make the case that viruses too must separate their genomes from each other at the cellular level. We propose a new hypothesis postulating how separation of viral genomes can be realized in virus-infected cells and chronicle the published studies in support of this hypothesis. While most of the relevant studies centered on single-stranded, plus-strand RNA [(+)RNA] viruses, the general principle on which our hypothesis is based may very well be applicable to viruses with other forms of genomes. The goals of this review are to call readers' attention to this unorthodox idea, to inspire more rigorous testing of this idea using different virus systems, and to explore novel virus control strategies that subvert this evolutionary arrangement.

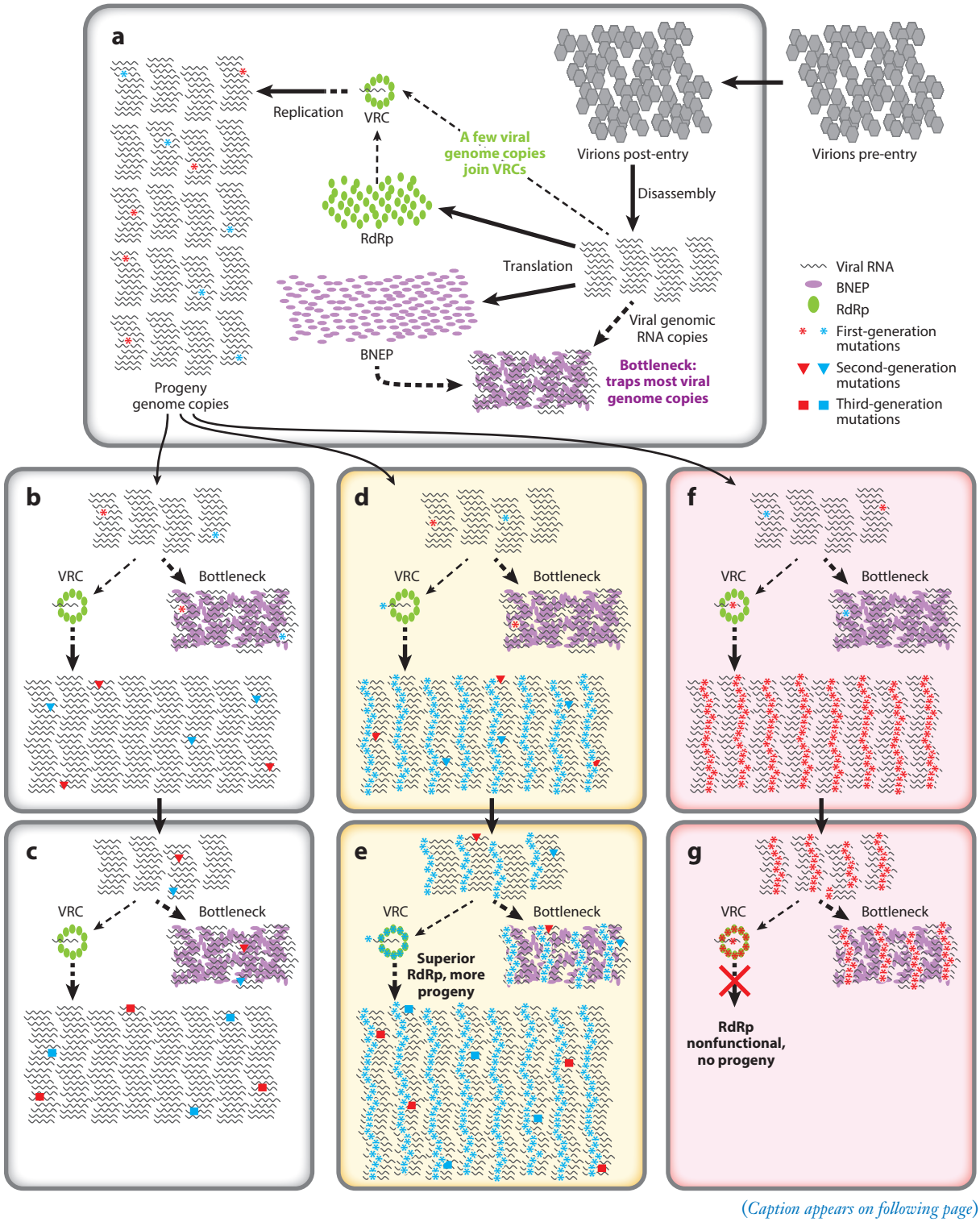
2. THE BIAS HYPOTHESIS

The basic concept of the Bottleneck, Isolate, Amplify, Select (BIAS) hypothesis is illustrated in **Figure 1*a***. It postulates that successful viruses encode strategies that limit the number of reproducing (replicating) genome copies in each infected cell to low single digits, often as low as one. Such strategies create the intracellular environment in which the reproducing viral genomes are separated from each other, allowing them to be acted upon by natural selection. Various prototypes of the BIAS hypothesis were proposed in several earlier publications (6–8). The version presented here reflects our latest refinement. Briefly, the revised hypothesis places more emphasis on the natural selection imperative of the BIAS arrangement. We wish to stress that the BIAS hypothesis in the current form applies only to (+)RNA viruses.

According to the BIAS hypothesis, (+)RNA viruses limit the number of replicating genomes per cell by establishing intracellular viral population bottlenecks. More specifically, experimental evidence suggests that most cells infected by a (+)RNA virus internalize hundreds, sometimes thousands, of copies of the viral genome (9). Nonetheless, the vast majority of the internalized genome copies are prevented from initiating replication by intracellular population bottlenecks and hence are genetic dead ends. As a result, the few genomes that do escape from the bottlenecks become isolated from each other in separate cells. It is worth emphasizing that the escape is stochastic and thus incapable of favoring advantageous genotypes on its own. As depicted in **Figure 1*b–g***, it is the bottleneck-enabled isolation that subsequently facilitates both positive and purifying selections acting on the isolated viral genome copies.

Two additional clarifications are in order here. First, claiming most cells are invaded by large numbers of viral genome copies does not dismiss the possibility that a small percentage of cells can become infected by just a few virions. Indeed, we foresee that in any given host individual, the first cells contracting a virus could very well receive just a few virions. However, initial success in these early cells is bound to yield millions of progeny virions that then infect the remaining susceptible cells in the same host en masse. Second, the majority of internalized viral genome copies, although blocked from replication by intracellular bottlenecks, nonetheless contribute to the success of the escaped few. Indeed, the BIAS hypothesis argues that the bottlenecked genome copies are themselves indispensable for the establishment of intracellular viral population bottlenecks, as shown in **Figure 1*a*** and explained next.

How exactly are the bottlenecks established? Emerging evidence suggests that, among the viral proteins directly translatable from the genome of a (+)RNA virus, at least one of them possesses the capacity to repress genome replication of the same virus, especially when its intracellular



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Schematic depiction of the BIAS model. (*a*) The journey of a (+)RNA virus population starting from the right. Upon cellular entry and particle disassembly, the many copies of viral RNA (*wavy lines*) template the translation of BNEP and RdRp (as a representative of replication proteins), denoted as purple and green ovals, respectively. BNEPs coalesce to form the multimeric assembly that traps the majority of internalized genomes. In the meantime, RdRps are assembled into VRCs that recruit the few genomes that escaped from the bottleneck. The ensuing replication produces many more genome copies, some of which contain new consequential mutations that affect RdRp function (*blue* and *red stars*). (*b–g*) Populations of viral genomes amplified in panel *a* enter cells in panels *b*, *d*, and *f*, where they meet different fates. In these cells new population bottlenecks are erected with BNEPs translated from incoming genome copies, allowing few genomes to escape. In the cell in panel *b*, the escaped genome is phenotypically similar to wild-type, yielding descendants that are mostly wild type but some with new mutations (*blue* and *red triangles*). The descendant population further continues a similar fate in the cell in panel *c*. In the cell in panel *d*, the escaped genome harbors a beneficial mutation, yielding descendants that all contain the beneficial mutation. Upon entering the cell in panel *e*, the incoming mutation-containing genome copies all translate the superior RdRps, which together amplify the bottleneck-escaping copy, also harboring the beneficial mutation, to higher numbers than wild type (compare *panels c* and *e*). In the cell in panel *f*, the escaped genome contains a lethal RdRp mutation that can nevertheless be replicated with RdRps translated from the incoming genome copies. However, upon entering the cell in panel *g*, the uniformly defective population, bottlenecked or not, loses the ability to replicate, thereby leading to the purge of the lethal mutation. Abbreviations: (+)RNA, plus-strand RNA; BIAS, Bottleneck, Isolate, Amplify, Select; BNEP, bottleneck-enforcing protein; RdRp, RNA-dependent RNA polymerase; VRC, viral replication complex. Figure adapted from Reference 8 (CC BY-NC 4.0).

concentration is high (**Figure 1*a***). Such proteins are given the name bottleneck-enforcing proteins or BNEPs (8). It should be noted that at least for two viruses—turnip crinkle virus (TCV) and tobacco mosaic virus (TMV)—the BNEP is the same protein as the virus-encoded auxiliary replication protein (ARP) (3, 6; C. Perdoncini-Carvalho, J. Han, and F. Qu, unpublished data). ARPs of these viruses were previously determined as being required for viral genome replication (10, 11). Therefore, for TCV and TMV, a single protein exerts two opposite functions: facilitating replication (of a few) as an ARP and repressing replication (of most) as a BNEP. Existing evidence suggests that the ARP function is most active when the protein concentration is low, whereas the BNEP function is most potent when the protein concentration is high.

We now consider cells invaded by numerous genome copies of a (+)RNA virus, as depicted in **Figure 1*a***. Before any of the viral genome copies start to replicate themselves, they must serve as messenger RNA (mRNA) to direct the translation of viral proteins such as RNA-dependent RNA polymerase (RdRp) and ARP, as well as BNEP (**Figure 1*a***). Remember that nearly all of the internalized viral genomes can serve as mRNA to template the translation of these proteins. Thus, it would not be difficult for the BNEP concentration to rise quickly, enabling strong intracellular population bottlenecks that sequester the majority of internalized viral genome copies (**Figure 1*a***). However, at this time the bottlenecks may still be slightly leaky, permitting occasional stochastic escape of a few viral genome copies. Mechanistically, such bottlenecks probably comprise multimeric assemblies of BNEPs that sequester viral replication proteins such as ARP and/or genomic RNA, preventing them from participating in replication (see Section 5.5).

We should not forget that while BNEPs are being translated, viral replication proteins such as RdRp and ARP are also being translated from the internalized viral genomic RNAs. Indeed, in TCV and TMV, the BNEP is itself part of the replication machinery, acting as an ARP. Therefore, in parallel with intracellular population bottlenecks, virus replication factories, also known as viral replication complexes (VRCs) or replication organelles, are also being busily constructed (**Figure 1*a***). This arrangement guarantees that any bottleneck-escaping genome copies are immediately admitted by VRCs to embark on replication.

Once active replication of the escaped viral genomes commences, thousands of new genome copies are produced in short order. These progeny genomes could then template massive translation of more BNEPs, further tightening the intracellular population bottleneck. Counterintuitively, this tightening would mean most, if not all, of the progeny genome copies would be denied

re-replication in the cells of their own genesis. Therefore, the BIAS hypothesis predicts that most viruses undergo just a single cycle of replication in each cell. Extending from this logic, secondary invasion of the same cells by the same virus, known as superinfection, would expose the superinfecting viral genomes to existing intracellular population bottlenecks, causing them to be arrested in the same manner as progeny genomes. Thus, the BIAS hypothesis provides a compelling explanation for the cellular-level superinfection exclusion (SIE) (12–16).

We now go back to the first cells of a new host individual falling victim to a virus, which may internalize just a few viral genome copies. This scenario is worth discussing as it is relevant to real-world infections. For example, plant viruses transmitted by insect vectors encounter frequent bottlenecks during the transmission step so that only a few virus particles succeed in infecting new plants (17, 18). In these plants, the earliest cells becoming infected by the viruses are unlikely to internalize many copies of the viral genomes. In these cells, the amount of BNEPs translated from the few internalized viral genomes may be insufficient for the intracellular bottlenecks to be established right away. Consequently, the few internalized genomes will probably all have the chance to replicate themselves. Furthermore, very few of the descendant genomes might replicate for another cycle in the same cell. However, keep in mind that in these cells it will likely also take a longer time for viral replication to initiate, as viral replication proteins also must be translated from very few genomic RNA copies. This would in turn give more chance for the host cell's innate immunity to react to virus attack. As a result, the viral success rate in these cells would probably be lower than for the cells entered by larger amounts of viral genomes.

3. SPECIFIC SCENARIOS OF THE BIAS ARRANGEMENT

Wait! An attentive reader might protest by now: "Did you say that both the establishment of population bottlenecks and the amplification of the escaped genomes are made possible by BNEPs, ARPs, and RdRps translated from the genomes that are excluded from replication and hence are genetic dead ends? How can natural selection act on BNEPs, ARPs, or RdRps encoded by the escaped genome if they do not even participate in replication, leaving them no chance to manifest their phenotypes?" The answer is that selection for these proteins occurs in the new cells entered by the immediate descendants of the bottleneck-isolated viral genomes.

To better illustrate this point, let us turn to **Figure 1*b*,*d*,*f*** depicting three cells that each internalize a pool of descendant viral genomes pre-amplified in the cell in **Figure 1*a***. These genomes all contain random mutations, but only a few of them are expected to have measurable phenotypic effects. For simplicity, we presume these mutations either enhance or abolish RdRp activities. In the cells in **Figure 1*b*,*c***, the viral genome escaping bottlenecks happens to be phenotypically wild type. As a result, replication repeats in successive new cells at the wild-type rate. Although new mutations emerge in every replication cycle (**Figure 1*b*,*c***), these mutations, be they advantageous or lethal, are sequestered by the bottlenecks and hence are not amplified.

In the next scenario, the bottleneck-escaping genome in the cell in **Figure 1*d*** happens to harbor a mutation that enhances RdRp activity. Despite the presence of this mutation, the mutant genome is not amplified to higher numbers because in this cell the RdRps responsible for replication are translated from the internalized genomes and hence are still mostly wild type. Nevertheless, the newly replicated genomes all contain the beneficial mutation and, upon entering the cell in **Figure 1*e***, translate RdRps that are uniformly superior. Together these superior RdRps amplify the new bottleneck-escaping genome, which harbors the beneficial mutation as well, to higher numbers than in the cell in **Figure 1*c***, thus embarking on the virtuous cycles of positive selection.

In the third scenario, the bottleneck-escaping genome in the cell in **Figure 1*f*** contains a lethal RdRp mutation. While in this cell, the mutant genome is still amplified with the mostly wild-type

RdRps translated from the internalized genome pool. However, its next-generation descendants, upon entering the cell in **Figure 1*g***, can no longer replicate because now all RdRps are defective. As a result, the lethal mutation is purged from the viral population through purifying selection. To summarize, the intracellular population bottleneck acts as a quality inspector that constantly rewards viral genomes with beneficial mutations and discards those with lethal mutations.

4. EVOLUTIONARY IMPERATIVE OF THE BIAS ARRANGEMENT

We just laid out the case that the bottleneck-enabled cellular-level isolation of viral genomes creates the environment in which viruses can be acted upon by natural selection in order to proliferate beneficial mutations and purge lethal ones. Extending from this, we advocate that the BIAS arrangement is itself positively selected. To arrive at this idea, we have only to imagine how a virus mutant would fare in real-world infections, should it lack the ability to isolate its genome copies in separate cells through intracellular population bottlenecks. As a result, multiple genome copies of this mutant would replicate in the same cell. Defective genome copies that incur lethal mutations in protein-coding genes would be able to borrow the corresponding proteins translated from sister genomes in the same cell. Indeed, defective genome copies might even gain an advantage by forgoing the protein-coding capacity, as evidenced by the frequent emergence of defective interfering RNAs in many (+)RNA virus infections (19).

More ominously, similar lethal mutations continuously emerge through repetition of error-prone replication, in the same and subsequently infected cells. Indeed, measurements made with several (+)RNA viruses suggest that a typical viral RdRp introduces approximately one error for every 10^4 nucleotides it incorporates into an RNA chain (a 10^{-4} error rate) (20–22). Considering that a progeny genome of a (+)RNA virus is copied from a negative-strand RNA intermediate that is in turn copied from a (+)RNA parent, a 10^{-4} error rate would mean that any (+)RNA virus genome larger than 5,000 nucleotides would contain at least one error when compared to its parent. Thus, every single new genome is different from the parental genome at a minimum of one nucleotide position after just one replication cycle. Even assuming 1% of the mutations are lethal to the virus, it would take no more than 100 replication cycles to corrupt all genome copies. Put differently, if a genome of a (+)RNA virus is allowed to replicate for 5 cycles per cell (4), nearly all of its descendants would be defective after replicating in 20 consecutive cells. Therefore, a virus mutant lacking the means to constantly surveil its own progeny genome copies through natural selection has no chance to compete with variants that do encode such capacities. One can further speculate that in a population of a virus that lacks the bottlenecking function, mutant genomes could emerge that gain this function. Once this occurs, the new mutants would quickly outcompete all other variants. In short, the ability to bottleneck its own genome copies, by enabling constant quality surveillance of viral genomes, is itself a virus-encoded trait favored by natural selection.

5. EVIDENCE IN SUPPORT OF THE BIAS HYPOTHESIS

5.1. Cross Protection and Superinfection Exclusion as Manifestations of Viral Population Bottlenecks

The earliest hint of the existence of viral population bottlenecks in infected hosts can be traced back to 1929, when McKinney found that pre-inoculating tobacco plants with a mild TMV isolate causing green symptoms prevented subsequent infection of the same plants by a more severe TMV isolate associated with yellow symptoms (23). The enormous amount of follow-up research on this cross protection phenomenon and its adoption as a means to manage virus diseases of crop

plants has been extensively reviewed by Ziebell and Carr (24), and hence is not repeated here. Various mechanistic theories have been proposed to explain cross protection, yet none of them gained undisputed experimental support. Especially worth mentioning is the invocation of RNA silencing as a mechanism, first proposed by Baulcombe (25). However, this too has been rejected by investigations carried out in several independent groups, with numerous different viruses along with mutant plants lacking key RNA silencing components (26, 27).

In contrast, the BIAS hypothesis provides a satisfactory explanation for cross protection as well as the related SIE phenomenon (6–8, 28, 29). It should be noted that in both cross protection and SIE, rejection of the superinfecting viruses depends on active replication of the primary virus in the host plants. Furthermore, the superinfecting virus must be sufficiently homologous to the primary virus in order to trigger SIE. Indeed, SIE does not occur between many distant isolates of citrus tristeza virus, a virus that co-evolved with its hosts in diverse geographical environments across the globe (28, 29). These characteristics of SIE could be explained by virus-encoded intracellular population bottlenecks that prevent the descendants of the primary virus from replicating in the same cell and recognize the highly similar superinfectors as among the descendants.

In contrast to organismal and tissue-level SIEs that were extensively examined in plant virus–infected plants, cellular-level SIEs were more carefully studied in animal virus–infected cell cultures (12–16). It is through these studies that a clear link between viral replication and SIE manifestation was first established. For instance, Tscherne and colleagues (14) found that SIE against hepatitis C virus occurred at a step downstream of protein translation from the primary virus genome and can be reversed when the translation of the primary genome was blocked. Separately, Zou and colleagues (13) demonstrated that SIE against West Nile virus (WNV) depended on the replication of the primary virus genome and WNV mutants overcoming SIE mapped to viral nonstructural proteins (nsPs) required for replication. Together these findings are consistent with the BIAS hypothesis postulating the existence of an intracellular population bottleneck limiting the number of viral genomes allowed to replicate per cell.

5.2. Tissue-Level Population Bottlenecks of Plus-Strand RNA Viruses in Plants and Arthropods

While intracellular viral population bottlenecks have only recently been unveiled, systemic movement-level bottlenecks of plant virus populations have been known for more than 20 years (30–32). The earlier studies were mostly carried out before the era of high-throughput sequencing and hence had to rely on viral variants that were easily distinguishable with nucleic acid hybridization, restriction enzyme digestion, or fluorescent protein markers (33). At least three different viruses—TMV, cucumber mosaic virus, and wheat streak mosaic virus—were examined, leading to the conclusion that infections in systemic leaves were initiated by no more than 20 different virus particles and sometimes as few as 5 (30–32, 34).

We now know that these conclusions may not be entirely accurate. Zhang and colleagues (27) later determined that when a plant leaf was inoculated with a mixed inoculum containing multiple similarly competent genotypes of the same virus, accumulation of a single genotype in a systemic leaf depended on two interrelated factors (27). The first was the relative time needed for a locally amplified genotype lineage (rather than a single virus particle!) to reach the systemic leaf. As schematically illustrated in **Figure 2*a***, the various viral genotypes in the mixed inoculum first replicated in spatially separated cells in the inoculated leaf and spread cell to cell to form independent primary infection foci (**Figure 2*a***). Simply by chance, some of the foci were physically closer to vascular bundles in Class II veins than others, allowing them to beat others in supplying large numbers of progeny genomes to the systemic leaves (**Figure 2*b***). As a result, infection in a

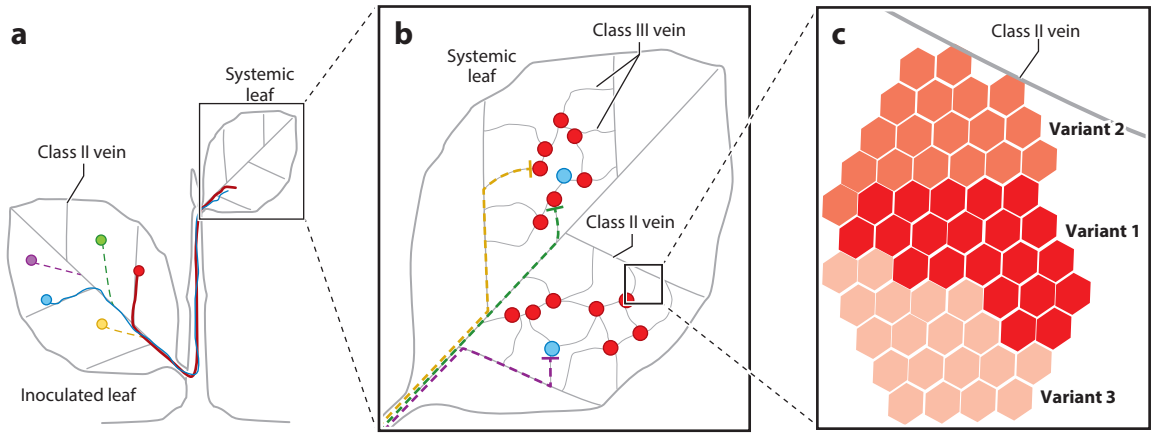


Figure 2

Model for plant virus population bottlenecking occurring at the systemic movement stage. (*a*) Five different viral variants initiate replication and cell-to-cell movement in an inoculated leaf to form infection foci denoted as colored dots. Note that the red dot and the blue dot, to a lesser extent, sit adjacent to a Class II vein (69). As a result, the red dot is likely to be the first to load the vascular bundles with its contents. Consequently, the systemic leaves are likely to be dominated by descendants of this variant. (*b*) Most of the exit sites (Class III vein junctions) in the systemic leaf are occupied by the early-arriving red variant and a few by the blue variant. Successful replication of these variants in these cells blocks late-arriving variants (*dashed lines*) through SIE. (*c*) Virus replication in the systemic leaf colonies gives rise to multiple new variants that initiate separate infections in different cells (denoted as *hexagons* in different *shades of red*). Their cell-to-cell spread, constrained by variants in neighboring cells through SIE, forms their own outward-expanding cell clusters, further allowing some variants (e.g., variant 2) to reach vascular bundles earlier than others. Abbreviation: SIE, superinfection exclusion. Figure adapted from Reference 27 (CC BY 4.0).

systemic leaf appeared to begin with one particle but in reality entailed many viral genome copies being released from one infection focus in the inoculated leaf, all descending from a single primary genotype. Note that such good fortune would fall on a different genotype in a different plant, in an unpredictable fashion, which accounted for the stochasticity of the reported population bottleneck examples (30–32, 34, 35).

The second and determining factor was SIE or, as we described earlier, the actively enforced intracellular population bottlenecks. Specifically, infection of systemic leaf cells by the early-arriving genotype lineage established population bottlenecks in each of the cells, blocking the replication of even their own immediate descendants and thereby collaterally excluding individuals of the later-arriving genotype populations from replication (27) (**Figure 2*b***). In short, the population bottlenecks observed at the systemic movement stage of plant virus infections likely reflected the combined effect of early colonization of systemic leaves by a descendant population of a single (or very few) genotype and subsequently active intracellular SIE against the late-arriving genotypes.

Further expanding this line of thought leads to interesting predictions. Even if all virions that arrived at a systemic leaf descended exclusively from one single genotype (**Figure 2*b***), they could still differ from each other due to new mutations incurred during replication. Once a few of the new variants succeeded in establishing infections in new cells (**Figure 2*c***), they would follow separate cell-to-cell movement paths by excluding each other wherever their infected cells border each other (**Figure 2*c***). This prediction is consistent with studies showing that simultaneous colonization of a systemic leaf by two or more discernable variants, such as viral variants labeled with two fluorescent proteins, caused them to form distinct single-variant cell clusters that bordered each other (33, 35–39). To extrapolate even further, such spatial separation of different variants in systemic leaves produces new winners and losers: As shown in **Figure 2*c***, the variant b cluster, by

virtue of merging into vascular bundles ahead of variants a and c, is predicted to contribute the bulk of viral genomes responsible for infections in younger leaves.

This rationale could be applied to the population dynamics of WNV in infected mosquitoes (40, 41). As the WNV infection spread through four different anatomically distinct tissues of a mosquito—midgut, hemolymph, salivary gland, and finally saliva—the WNV populations diversified within the same tissue but then became bottlenecked in the next tissue. According to the BIAS hypothesis, new WNV genotypes arising from intra-tissue diversification embarked on bottlenecked replication in successive cells to form mutually exclusive, spatially separate clusters of infected cells. Some of the cell clusters are bound to be closer to inter-tissue connections than others and hence supply most of the viruses for the next tissue type. Their early colonization there in turn blocks superinfecting late arrivals. Dominance of the new tissue type by viruses derived from very few cell clusters in the preceding tissue type would give rise to the appearance of tissue-to-tissue bottlenecking (40, 41). This explanation is also consistent with a study by Frost and colleagues (42) showing human immunodeficiency virus 1 (HIV-1) forming spatially discrete subpopulations in the spleen of infected individuals.

5.3. Invasion of Single Cells by Multiple Viruses

If intracellular population bottlenecks act to isolate viral genomes and facilitate natural selection acting on viruses, cells of naturally infected animals or plants must be routinely invaded by large numbers of virions or viral genomes. Consistent with this idea, virologists are discovering that many viruses invade cells in the form of so-called collective infectious units (CIUs) (9, 43). CIUs take many forms. HIV, for example, spreads from infected to uninfected T cells through intercellular connections known as virological synapses that transmit massive amounts of virions between two cells (44–48). On the other hand, poliovirus and coxsackievirus particles were found to be nonlytically released from infected cells in the form of lipid membrane vesicles containing dozens of virions (49, 50). Such vesicles subsequently fuse with uninfected cells to deliver the enclosed virions en bloc (50). Separately, respiratory syncytial virus induces fusion of the infected cell membrane with that of neighboring cells to form giant multinucleated cells (51). Still other viruses connect infected and uninfected cells through tubular or filamentous extensions (52). Such multi-genome transmission is likely to be a more efficient mode of viral intercellular spread inside infected individuals (53, 54). Importantly, plant viruses spread cell to cell through plasmodesmata channels modified by virus-encoded movement proteins. Such modified intercellular channels are thought to shuffle large numbers of virions or viral genomes between adjacent cells.

5.4. Direct Evidence of Intracellular Viral Population Bottlenecks

Miyashita and colleagues (55) provided the first direct evidence of intracellular population bottlenecks for a (+)RNA virus. Their virus population was based on tomato mosaic virus (ToMV), a (+)RNA virus belonging to genus *Tobamovirus* and family *Virgaviridae*, for which infectious RNA can be synthesized in vitro. To construct a ToMV population, the authors inserted a 10-nucleotide-long nonviral sequence within the 3′ untranslated region of the ToMV genome, at a position known to not affect viral infectivity. Importantly, each of the 10 inserted nucleotides was randomized, giving rise to a ToMV population of 4^{10} variants (1,048,576). This ToMV population was then used to infect individual wall-less plant cells, called protoplasts. It was determined that at least 5,000 infectious RNA molecules entered each of the protoplasts; 15 infected protoplasts were then randomly selected to count the number of replicating variants in each protoplast and their relative multiplication levels. The most important take-home message is that each protoplast cell replicated no more than seven variants, thus revealing stunningly narrow intracellular

population bottlenecks. Further highlighting the stochastic nature of the bottlenecks is that the identities of the variants isolated from the 15 cells were all different from each other, indicating the absence of immediate positive selection. Most importantly, the relative accumulation levels of variants coinhabiting the same cell differed dramatically, with 8 of the 15 cells dominated by a single variant.

Zhang and colleagues (6) took a different approach to examine intracellular population bottlenecks of TCV, a member of the genus *Betacarmovirus*, family *Tombusviridae*. They loaded cells of *Nicotiana benthamiana* plants with complementary DNAs (cDNAs) of two TCV replicons, TCV_sg2G and TCV_sg2R (**Figure 3*a***), using a procedure known as agro-infiltration (56, 57), which is capable of delivering multiple constructs into the same cell at near 100% codelivery rates. These cDNAs were equipped with the duplicated 35S promoter (2X35S) of cauliflower mosaic virus, powering strong transcription by plant RNA polymerase II, producing thousands of replicon RNA copies inside the cells (58). Replication of TCV_sg2G and TCV_sg2R would lead to the expression of green fluorescent protein (GFP) and red fluorescent monomeric Cherry (mCherry), respectively, providing easily scorable visual markers.

Given that thousands of copies of TCV_sg2G and TCV_sg2R RNAs were transcribed in each cell, one would expect that if just two of the replicon RNA copies succeeded in launching replication (an intracellular bottleneck size of two), up to 50% of fluorescent cells would express both GFP and mCherry [based on the formula $(a + b)^2 = a^2 + 2ab + b^2$, with 2ab representing the fraction of cells replicating both variants]. However, in actual experiments fewer than 0.1% of fluorescent cells expressed both GFP and mCherry, despite the fact that cells expressing GFP or mCherry alone were equally abundant and frequently adjacent to each other (**Figure 3*b***). Furthermore, parallel experiments involving codelivery of TCV_sg2G and a different virus expressing mCherry (carnation mottle virus, CarMV_sg2R; **Figure 3**) caused more than 80% of the cells to express both GFP and mCherry, indicating that the TCV-enforced bottlenecks allowed just one TCV genome to escape, and they strictly targeted TCV genomes only.

5.5. Active Enforcement of Intracellular Population Bottlenecks by Virus-Encoded Bottleneck-Enforcing Proteins

How were intracellular population bottlenecks established? It turned out that the TCV-encoded p28 protein, when present at high intracellular concentrations, was alone sufficient to repress TCV replication (6, 56). Note that p28 is also the ARP of TCV (10). It is encoded on the genomic RNA and thus directly translatable from all TCV genomes in the infected cells. As a result, rapid transcription of TCV genomic RNA in agro-infiltrated cells, propelled by the strong 2X35S promoter, would have provided abundant templates for p28 translation. The resulting high intracellular levels of p28 protein would fuel the swift establishment of the intracellular population bottlenecks that in turn block the replication of most of the TCV genomic RNA copies (**Figure 1**).

To determine how p28 blocked TCV replication, Zhang and colleagues (6, 56) created two different p28 mutants—G11-p28 and p28-mCherry (**Figure 4*a***). In G11-p28, the full-length p28 protein was fused at the N terminus with the last (the eleventh) β-strand of GFP, hence the name G11. As a result, the intracellular distribution of G11-p28 can be tracked by coexpressing G1–10, the remaining 10 β-strands of GFP (59, 60). G11-p28 accumulated to relatively low levels in plant cells but was replication competent as it readily complemented the replication of a defective TCV lacking its own p28 (6). G11-p28 was diffusely distributed in cells coexpressing G1–10 (**Figure 4*b***). By contrast, p28-mCherry had the mCherry fused to the C terminus of the p28 protein (**Figure 4*a***). It accumulated to high levels in plant cells but completely blocked TCV replication (6). Notably, it formed discrete, intensely fluorescent intracellular foci, indicative of

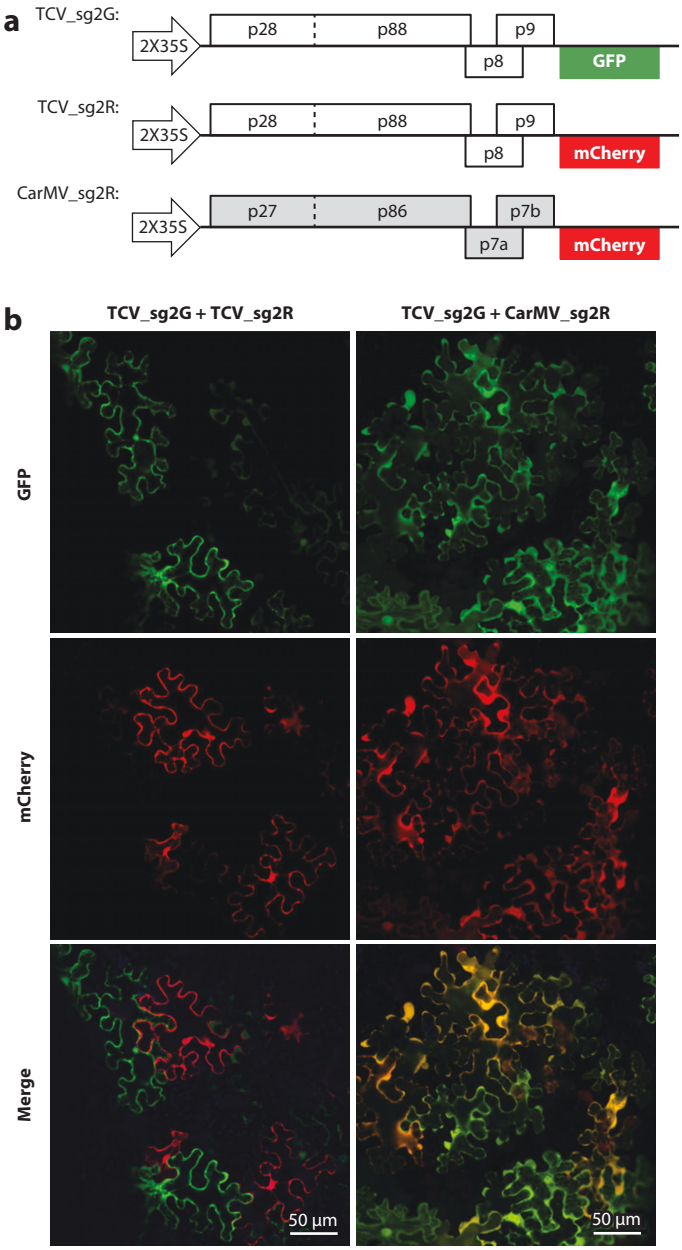


Figure 3

Intracellular population bottlenecks of TCV replicons. (*a*) Three viral replicon constructs, TCV_sg2G, TCV_sg2R, and CarMV_sg2R. Note that GFP and mCherry expression from these replicons is replication dependent. (*b*) Mutual exclusion of TCV_sg2G and TCV_sg2R (*left column*) and mutual accommodation of TCV_sg2G and CarMV_sg2R (*right column*) in *Nicotiana benthamiana* cells. Abbreviations: CarMV, carnation mottle virus; GFP, green fluorescent protein; mCherry, monomeric Cherry; TCV, turnip crinkle virus. Figure adapted from Reference 6 (CC BY 4.0).

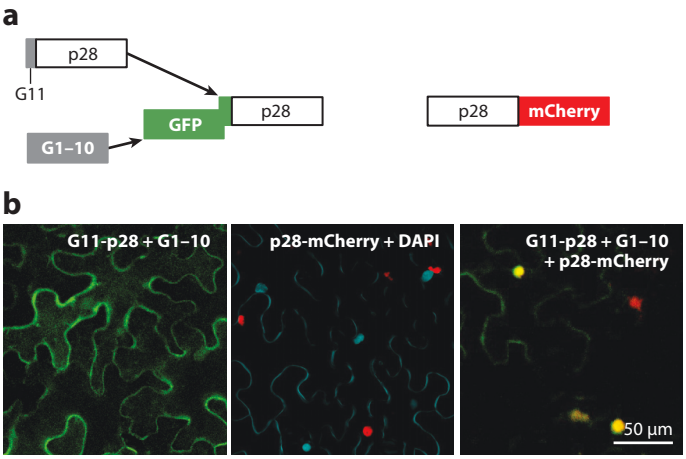


Figure 4

A diffusely distributed, replication-competent p28 mutant trapped in the multimeric assemblies of a replication-blocking p28 mutant. (*a*) The G11-p28 and p28-mCherry mutants of p28. G11-p28 harbors an N-terminal G11 fusion, whose intracellular distribution can be tracked by coexpressing G1–10. p28-mCherry contains a C-terminal mCherry fusion. (*b*) The intracellular distribution patterns of the two mutants, alone (*left* and *middle*) and combined (*right*). Abbreviations: GFP, green fluorescent protein; mCherry, monomeric Cherry. Figure adapted from Reference 6 (CC BY 4.0).

multimeric protein assemblies (**Figure 4*b***). Strikingly, when G11-p28 and p28-mCherry were coexpressed in the same cells, G11-p28 (plus G-10) lost its diffuse distribution. Instead, most of the green fluorescence became trapped in the p28-mCherry foci (**Figure 4*b***). These results strongly suggest that the repressive state of p28 took the form of the multimeric assemblies. Once formed, such protein assemblies trap replication-competent p28 molecules, sequestering the latter away from the VRCs. These and other observations together (6–8, 56) uncover a novel molecular switch that routes TCV p28 to two opposite functional states based on its concentration in the cell, providing a simple mechanism for p28-mediated intracellular bottlenecking of TCV populations.

6. TESTABLE PREDICTIONS OF THE BIAS HYPOTHESIS

Many details of the BIAS arrangement remain to be resolved. Nevertheless, the current BIAS model yields several testable predictions that depart from currently prevalent views. Below we discuss three immediate predictions, the careful testing of which will subject the BIAS hypothesis to thorough scrutiny.

6.1. *Cis*-Preference of Viral Nonstructural Proteins

Cis-preference refers to the phenomenon that some virus-encoded nsPs, especially those involved in genome replication of (+)RNA viruses, appear to preferentially or exclusively serve the very genomic RNA copies from which they are translated (10, 61–65). This phenomenon was frequently observed when researchers tried to use one viral genome that encodes a functional nsP to complement a mutant genome encoding a null mutant of the same nsP. Such complementation frequently failed, leaving the former genome to be the sole one that replicated. Various models were proposed to explain this phenomenon. The most recent model (63) asserted a tight cotranslational binding of the nsP to the same viral RNA from which it is translated. However, this model raises several questions. For example, do the many copies of nsP translated from the same viral

RNA all engage in such tight interaction? Do they bind to the same RNA motif? How can the nsP function with subgenomic RNAs that do not translate this nsP? How about viruses with multiple genome segments, only one of which templates the translation of the *cis*-preferential nsP?

The BIAS model postulates that the particular nsP is also a BNEP, thus bottlenecking most viral genomes, especially when it is present at high concentrations in the cells. The genome translating the functional nsP more frequently escapes from the bottleneck because it has ample access to low concentration nsP, likely at the very early stage of nsP translation. By contrast, the genome encoding defective nsP must wait for the nsP to be translated from its complementation partner and hence more frequently encounters the high-concentration BNEP state of the nsP. A testable prediction from this idea is that one should be able to identify a mutant nsP that abolishes mutual SIE of the parental virus, and such an nsP mutant would also lose *cis*-preference, hence gaining the ability of *trans*-complementation.

6.2. Reduced Viral Fitness in Small Host Organisms

We stressed earlier that the intracellular population bottlenecks are themselves incapable of picking winners. The viral genomes that are better at survival and reproduction (hence more fit) win out by producing more viral genome copies cell after cell, eventually becoming the dominant variants in the virus population inhabiting a given host individual. It then follows that if the host individual does not possess a sufficient number of susceptible cells, the more fit genomes might not have the chance to reach dominance, due to insufficient numbers of replication cycles. A testable prediction here is that a virus infecting *Caenorhabditis elegans*, which has no more than 1,031 cells in the entire body, will likely yield a high proportion of descendants that are less fit. This prediction is consistent with the study by Grubaugh and colleagues (40) showing that WNV populations passing through mosquitoes suffered fitness losses and retained deleterious mutations. Given that WNV must spread from one tissue type to another sequentially, the number of cells in each of the tissues might be insufficient for positive selection to reach its full potential.

6.3. One Replication Cycle Per Cell?

We predicted earlier that most of the susceptible cells in an infected individual are likely entered by large numbers of viral genomes, leading to the swift establishment of intracellular population bottlenecks that permit a few genomes to undergo replication for a single cycle. Given that RdRps of (+)RNA viruses incur replication errors at a relatively constant rate, we predict that progeny genomes in a virus-infected cell would contain a near-identical number of new errors. This prediction should now be testable, thanks to the single-cell, long-read sequencing techniques emerging lately (66–68).

7. CONCLUDING REMARKS

We have updated the BIAS hypothesis and chronicled the relevant studies done by many outstanding researchers that culminated in this hypothesis. The most compelling rationale for this hypothesis is that it adequately addresses the question of how (+)RNA viruses are acted upon by natural selection to proliferate beneficial mutations and to purge lethal ones. Many questions remain unanswered. For example, how do viruses begin replication with one or a few genomes per cell yet generate thousands of descendants through a single cycle replication? How does recombination between two closely related viruses occur if they cannot replicate in the same cell? We hope to call the attention of fellow virologists to this hypothesis and welcome rigorous debates and testing of its many predictions. We are confident that intensive examination of this subject will yield tangible solutions to viral diseases of humans, animals, and plants.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We apologize to the many authors whose works were omitted due to the word limit of this review. We thank members of the Qu laboratory for discussions and research assistance. We acknowledge the US Department of Agriculture Agricultural Research Service Corn and Soybean Virus Research lab for generous equipment sharing. Finally, we thank the National Science Foundation for partial financial support.

LITERATURE CITED

1. Dawkins R. 1989. *The Selfish Gene*. Oxford, UK: Oxford Univ. Press
2. Cook LM, Grant BS, Saccheri IJ, Mallet J. 2012. Selective bird predation on the peppered moth: the last experiment of Michael Majerus. *Biol. Lett.* 8:609–12
3. Robinson PS, Coorens THH, Palles C, Mitchell E, Abascal F, et al. 2021. Increased somatic mutation burdens in normal human cells due to defective DNA polymerases. *Nat. Genet.* 53:1434–42
4. Schulte MB, Draghi JA, Plotkin JB, Andino R. 2015. Experimentally guided models reveal replication principles that shape the mutation distribution of RNA viruses. *eLife* 4:e03753
5. Kang L, He G, Sharp AK, Wang X, Brown AM, et al. 2021. A selective sweep in the *Spike* gene has driven SARS-CoV-2 human adaptation. *Cell* 184:4392–400.e4
6. Zhang X-F, Sun R, Guo Q, Zhang S, Meulia T, et al. 2017. A self-perpetuating repressive state of a viral replication protein blocks superinfection by the same virus. *PLOS Pathog.* 13:e1006253
7. Zhang X-F, Zhang S, Guo Q, Sun R, Wei T, Qu F. 2018. A new mechanistic model for viral cross protection and superinfection exclusion. *Front. Plant Sci.* 9:40
8. Qu F, Zheng L, Zhang S, Sun R, Slot J, Miyashita S. 2020. Bottleneck, Isolate, Amplify, Select (BIAS) as a mechanistic framework for intracellular population dynamics of positive-sense RNA viruses. *Virus Evol.* 6(2):veaa086
9. Sanjuán R. 2017. Collective infectious units in viruses. *Trends Microbiol.* 25:402–12
10. White KA, Skuzeski JM, Li W, Wei N, Morris TJ. 1995. Immunodetection, expression strategy and complementation of turnip crinkle virus p28 and p88 replication components. *Virology* 211:525–34
11. Buck KW. 1999. Replication of tobacco mosaic virus RNA. *Philos. Trans. R. Soc. B* 354:613–27
12. Schaller T, Appel N, Koutsoudakis G, Kallis S, Lohmann V, et al. 2007. Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes. *J. Virol.* 81:4591–603
13. Zou G, Zhang B, Lim P-Y, Yuan Z, Bernard KA, Shi P-Y. 2009. Exclusion of West Nile virus superinfection through RNA replication. *J. Virol.* 83:11765–76
14. Tscherne DM, Evans MJ, von Hahn T, Jones CT, Stamataki Z, et al. 2007. Superinfection exclusion in cells infected with hepatitis C virus. *J. Virol.* 81:3693–703
15. Tscherne DM, Evans MJ, MacDonald MR, Rice CM. 2008. Transdominant inhibition of bovine viral diarrhea virus entry. *J. Virol.* 82:2427–36
16. Webster B, Ott M, Greene WC. 2013. Evasion of superinfection exclusion and elimination of primary viral RNA by an adapted strain of hepatitis C virus. *J. Virol.* 87:13354–69
17. Ali A, Li H, Schneider WL, Sherman DJ, Gray S, et al. 2006. Analysis of genetic bottlenecks during horizontal transmission of *Cucumber mosaic virus*. *J. Virol.* 80:8345–50
18. Moury B, Fabre F, Senoussi R. 2007. Estimation of the number of virus articles transmitted by an insect vector. *PNAS* 104:17891–96
19. Pathak KB, Nagy PD. 2009. Defective interfering RNAs: foes of viruses and friends of virologists. *Viruses* 1:895–919
20. Martinez F, Sardanyes J, Elena SF, Daros J-A. 2011. Dynamics of a plant RNA virus intracellular accumulation: stamping machine versus geometric replication. *Genetics* 188:637–46

21. Domingo E, Perales C. 2019. Viral quasispecies. *PLOS Genet*. 15:e1008271
22. Lauring AS, Andino R. 2010. Quasispecies theory and the behavior of RNA viruses. *PLOS Pathog*. 6:e1001005
23. McKinney HH. 1929. Mosaic diseases in the Canary Islands, West Africa, and Gibraltar. *J. Agric. Res.* 39:557–78
24. Ziebell H, Carr JP. 2010. Cross-protection: a century of mystery. *Adv. Virus Res.* 76:211–64
25. Baulcombe D. 2004. RNA silencing in plants. *Nature* 431:356–63
26. Ziebell H, Payne T, Berry JO, Walsh JA, Carr JP. 2007. A cucumber mosaic virus mutant lacking the 2b counter-defence protein gene provides protection against wild-type strains. *J. Gen. Virol.* 88:2862–71
27. Zhang X-F, Guo J, Zhang X, Meulia T, Paul P, et al. 2015. Random plant viral variants attain temporal advantages during systemic infections and in turn resist other variants of the same virus. *Sci. Rep.* 5:15346
28. Folimonova SY. 2012. Superinfection exclusion is an active virus-controlled function that requires a specific viral protein. *J. Virol.* 86:5554–61
29. Folimonova SY. 2013. Developing an understanding of cross-protection by *Citrus tristeza virus*. *Front. Microbiol.* 4:76
30. Sacristán S, Malpica JM, Fraile A, García-Arenal F. 2003. Estimation of population bottlenecks during systemic movement of *Tobacco mosaic virus* in tobacco plants. *J. Virol.* 77:9906–11
31. Li H, Roossinck MJ. 2004. Genetic bottlenecks reduce population variation in an experimental RNA virus population. *J. Virol.* 78:10582–87
32. Hall JS, French R, Hein GL, Morris TJ, Stenger DC. 2001. Three distinct mechanisms facilitate genetic isolation of sympatric wheat streak mosaic virus lineages. *Virology* 282:230–36
33. Dietrich C, Maiss E. 2003. Fluorescent labelling reveals spatial separation of potyvirus populations in mixed infected *Nicotiana benthamiana* plants. *J. Gen. Virol.* 84:2871–76
34. French R, Stenger DC. 2003. Evolution of wheat streak mosaic virus: Dynamics of population growth within plants may explain limited variation. *Annu. Rev. Phytopathol.* 41:199–214
35. Zwart MP, Daròs J-A, Elena SF. 2011. One is enough: *In vivo* effective population size is dose-dependent for a plant RNA virus. *PLOS Pathog*. 7:e1002122
36. Tatineni S, French R. 2016. The coat protein and NIa protease of two *Potyviridae* family members independently confer superinfection exclusion. *J. Virol.* 90:10886–905
37. Zhou X, Sun K, Zhou X, Jackson AO, Li Z. 2019. The matrix protein of a plant rhabdovirus mediates superinfection exclusion by inhibiting viral transcription. *J. Virol*. 93:e00680-19
38. Tromas N, Zwart MP, Lafforgue G, Elena SF. 2014. Within-host spatiotemporal dynamics of plant virus infection at the cellular level. *PLOS Genet*. 10:e1004186
39. Takahashi T, Sugawara T, Yamatsuta T, Isogai M, Natsuaki T, Yoshikawa N. 2007. Analysis of the spatial distribution of identical and two distinct virus populations differently labeled with cyan and yellow fluorescent proteins in coinfected plants. *Phytopathology* 97:1200–6
40. Grubaugh ND, Weger-Lucarelli J, Murrieta RA, Fauver JR, Garcia-Luna SM, et al. 2016. Genetic drift during systemic arbovirus infection of mosquito vectors leads to decreased relative fitness during host switching. *Cell Host Microbe* 19:481–92
41. Ciota AT, Ehrbar DJ, Van Slyke GA, Payne AF, Willsey GG, et al. 2012. Quantification of intrahost bottlenecks of *West Nile virus* in *Culex pipiens* mosquitoes using an artificial mutant swarm. *Infect. Genet. Evol.* 12:557–64
42. Frost SDW, Dumaurier M-J, Wain-Hobson S, Brown AJL. 2001. Genetic drift and within-host metapopulation dynamics of HIV-1 infection. *PNAS* 98:6975–80
43. Leeks A, Sanjuán R, West SA. 2019. The evolution of collective infectious units in viruses. *Virus Res.* 265:94–101
44. Chen P, Hübner W, Spinelli MA, Chen BK. 2007. Predominant mode of human immunodeficiency virus transfer between T cells is mediated by sustained Env-dependent neutralization-resistant virological synapses. *J. Virol.* 81:12582–95
45. Hübner W, McNerney GP, Chen P, Dale BM, Gordon RE, et al. 2009. Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science* 323:1743–47
46. Del Portillo A, Tripodi J, Najfeld V, Wodarz D, Levy DN, Chen BK. 2011. Multiploid inheritance of HIV-1 during cell-to-cell infection. *J. Virol.* 85:7169–76

47. Iwami S, Takeuchi JS, Nakaoka S, Mammano F, Clavel F, et al. 2015. Cell-to-cell infection by HIV contributes over half of virus infection. *eLife* 4:e08150
48. Law KM, Komarova NL, Yewdall AW, Lee RK, Herrera OL, et al. 2016. In vivo HIV-1 cell-to-cell transmission promotes multicopy micro-compartmentalized infection. *Cell Rep*. 15:2771–83
49. Robinson SM, Tsueng G, Sin J, Mangale V, Rahawi S, et al. 2014. Coxsackievirus B exits the host cell in shed microvesicles displaying autophagosomal markers. *PLOS Pathog*. 10:e1004045
50. Chen Y-H, Du W, Hagemeijer MC, Takvorian PM, Pau C, et al. 2015. Phosphatidylserine vesicles enable efficient en bloc transmission of enteroviruses. *Cell* 160:619–30
51. Cifuentes-Muñoz N, Dutch RE, Cattaneo R. 2018. Direct cell-to-cell transmission of respiratory viruses: the fast lanes. *PLOS Pathog*. 14:e1007015
52. Martinez MG, Kielian M. 2016. Intercellular extensions are induced by the alphavirus structural proteins and mediate virus transmission. *PLOS Pathog*. 12:e1006061
53. Feng Z, Hensley L, McKnight KL, Hu F, Madden V, et al. 2013. A pathogenic picornavirus acquires an envelope by hijacking cellular membranes. *Nature* 496:367–71
54. Santiana M, Ghosh S, Ho BA, Rajasekaran V, Du W-L, et al. 2018. Vesicle-cloaked virus clusters are optimal units for inter-organismal viral transmission. *Cell Host Microbe* 24:208–20.e8
55. Miyashita S, Ishibashi K, Kishino H, Ishikawa M. 2015. Viruses roll the dice: The stochastic behavior of viral genome molecules accelerates viral adaptation at the cell and tissue levels. *PLOS Biol*. 13:e1002094
56. Guo Q, Zhang S, Sun R, Yao X, Zhang X-F, et al. 2020. Superinfection exclusion by p28 of turnip crinkle virus is separable from its replication function. *Mol. Plant-Microbe Interact.* 33:364–75
57. Qu F, Ren T, Morris TJ. 2003. The coat protein of *Turnip crinkle virus* suppresses posttranscriptional gene silencing at an early initiation step. *J. Virol.* 77:511–22
58. Hull R, Covey SN, Dale P. 2000. Genetically modified plants and the 35S promoter: assessing the risks and enhancing the debate. *Microb. Ecol. Health Dis.* 12:1–5
59. Cabantous S, Terwilliger TC, Waldo GS. 2005. Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein. *Nat. Biotechnol.* 23:102–7
60. Cabantous S, Waldo GS. 2006. *In vivo* and *in vitro* protein solubility assays using split GFP. *Nat. Methods* 3:845–54
61. Novak JE, Kirkegaard K. 1994. Coupling between genome translation and replication in an RNA virus. *Genes Dev*. 8:1726–37
62. Okamoto K, Nagano H, Iwakawa H, Mizumoto H, Takeda A, et al. 2008. *cis*-Preferential requirement of a −1 frameshift product p88 for the replication of *Red clover necrotic mosaic virus* RNA1. *Virology* 375:205–12
63. Kawamura-Nagaya K, Ishibashi K, Huang Y-P, Miyashita S, Ishikawa M. 2014. Replication protein of tobacco mosaic virus cotranslationally binds the 5′ untranslated region of genomic RNA to enable viral replication. *PNAS* 111:E1620–28
64. Yi G, Gopinath K, Kao CC. 2007. Selective repression of translation by the brome mosaic virus 1a RNA replication protein. *J. Virol.* 81:1601
65. Lin J, Guo J, Finer J, Dorrance AE, Redinbaugh MG, Qu F. 2014. The *Bean pod mottle virus* RNA2-encoded 58-kilodalton protein P58 is required in *cis* for RNA2 accumulation. *J. Virol.* 88:3213–22
66. Brese RL, Gonzalez-Perez MP, Koch M, O'Connell O, Luzuriaga K, et al. 2018. Ultradeep single-molecule real-time sequencing of HIV envelope reveals complete compartmentalization of highly macrophage-tropic R5 proviral variants in brain and CXCR4-using variants in immune and peripheral tissues. *J. Neurovirol.* 24:439–53
67. Russell AB, Elshina E, Kowalsky JR, te Velthuis AJW, Bloom JD. 2019. Single-cell virus sequencing of influenza infections that trigger innate immunity. *J. Virol.* 93:e00500-19
68. Yamashita T, Takeda H, Takai A, Arasawa S, Nakamura F, et al. 2020. Single-molecular real-time deep sequencing reveals the dynamics of multi-drug resistant haplotypes and structural variations in the hepatitis C virus genome. *Sci. Rep.* 10:2651
69. Roberts AG, Cruz SS, Roberts IM, Prior DAM, Turgeon R, Oparka KJ. 1997. Phloem unloading in sink leaves of *Nicotiana benthamiana*: comparison of a fluorescent solute with a fluorescent virus. *Plant Cell* 9:1381–96