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Genetic Bottlenecks Reduce Population Variation in an Experimental RNA Virus Population

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Genetic bottlenecks are stochastic events that limit genetic variation in a population and result in founding populations that can lead to genetic drift. Evidence of past genetic bottlenecks in numerous biological systems, from mammals to viruses, has been described. In this study, we used an artificial population of *Cucumber mosaic virus* consisting of 12 restriction enzyme marker-bearing mutants. This population was inoculated onto young leaves of tobacco plants and monitored throughout the course of systemic infection. We show here that the genetic variation in a defined population of an RNA virus is significantly, stochastically, and reproducibly reduced during the systemic infection process, providing clear evidence of a genetic bottleneck.

Populations generate genetic variation over time, which can be specifically reduced by selection or stochastically reduced by genetic bottlenecks. After a bottleneck, a limited number of randomly selected individuals create a founding population, resulting in genetic drift. The occurrence of bottlenecks in nature has been inferred from the low genetic diversity of extant populations and extrapolation of an historical event (reviewed in reference 1).

Genetic bottlenecks may occur frequently during the natural life cycles of RNA viruses. Transmission events, both horizontal and vertical, and systemic infections represent events in the virus life cycle that may impose a bottleneck. A few studies of plant viruses have attempted to estimate the effect of bottlenecks by examining diversity in populations from systemically infected tissues (18, 40). In addition, a number of studies of animal and bacterial viruses have examined changes in the population structure after passage through artificial bottlenecks (4, 7, 32, 49), but naturally occurring bottlenecks have been poorly studied.

RNA viruses are characterized by the potential for a high degree of variability due to short generation times and error-prone replication (reviewed in reference 11), leading to populations known as quasispecies (14, 15). In spite of this potential, quasispecies variation in experimental evolution studies with plant viruses was significantly lower than what might be predicted (42).

Low genetic diversity may result from low mutation rates. However, a rough estimation of the mutation rate of *Tobacco mosaic virus* suggested that plant viruses were not significantly different from animal RNA viruses (27). Selection (predominantly negative) for maintaining a functional encoded protein or functional RNA structure is believed to play an important role in the elimination of deleterious variants (20, 34, 36). Population bottlenecks during transmission might also be an important factor for limiting the variation in a population. This phenomenon was implicated in some laboratory passage experiments (2, 23, 25) as well as some analyses of natural plant virus populations (3, 6, 17). However, there hasn't been any direct experimental evidence showing that naturally occurring bottlenecks play a role in the genetic structure of viral populations.

A comparative study of the mutation frequencies in tobacco protoplasts and systemic leaves of intact plants for both *Cucumber mosaic virus* (CMV) and *Tobacco mosaic virus* found that mutation frequencies were much higher in isolated plant cells (protoplasts) than in intact plants. In systemically infected leaves, both viruses contained some genomic regions that did not accumulate mutations to a detectable level, but the majority of the mutations were distributed randomly (43). These results suggested that systemic movement played a role in the limitation of mutation frequencies and that at least some of the limitation was stochastic.

To assess the role of bottlenecks that may limit quasispecies variation during systemic virus infection, we used a cDNA clone of CMV to create an artificial population consisting of restriction enzyme-bearing marker mutants. CMV is a tripartite single-stranded RNA virus with a very broad host range (33). Isogenic young tobacco plants were inoculated with a mixture of 12 mutants in equal proportion on the fifth true leaf, and both inoculated leaves and systemic leaves (the 8th and 15th) were analyzed for the presence of the each of the 12 marker mutants at 2, 10, and 15 days postinoculation (dpi), respectively. We found that systemic infection induced a significant bottleneck in the CMV population. This is the first description of an analysis of a defined population passing through a natural bottleneck, and it clearly demonstrates the role of bottlenecks on population structure.

MATERIALS AND METHODS

Viruses and plants. Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were inoculated at the five-leaf stage with transcripts generated from cDNA clones of the Fny strain of CMV (37) at a concentration of 500 μg/ml with viral RNA purified from infected plants (38) or with sap from infected plants. Clones of Fny CMV capable of producing infectious transcripts have been described previously (35, 36).

Construction of the artificial population. To construct an artificial population bearing specific restriction enzyme site markers, an alignment of 26 CMV-RNA

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TABLE 1. Artificial population of CMV RNA 3 marker mutants

Mutant strain(s) (mutant)[a]	Primer used[b]		Enzyme used
	Forward	Reverse	
G1505A/T1508C (*a*)	5′-GACCGTGG**A**TC**C**TATTACGG-3′	5′-CCGTAATA**G**GA**T**CCACGGTC-3′	BamHI
C1574A (*b*)	5′-GCTTGTTTCGCG**A**ATTCAAATTCG-3′	5′-CGAATTTGAAT**T**CGCGAAACAAGC-3′	NruI
T1592C (*c*)	5′-CGAGTTAA**C**CCTTTGCCG-3′	5′-CGGCAAAGG**G**TTAACTCG-3′	HpaI
A1853C (*d*)	5′-GGACGAGCT**C**GTACTTCATG-3′	5′-CATGAAGTAC**G**AGCTCGTCC-3′	SacI
T1865C (*e*)	5′-CTTCATGT**C**GACATCGAGC-3′	5′-GCTCGATGTC**G**ACATGAAG-3′	SalI
A1926G/A1928G (*f*)	5′-CGGAGGGAGGATCCC**GG**G**A**ACACGG-3′	5′-CCGTGTT**CC**CGGGATCCTCCCTCCG-3′	SmaI
A1928G (*g*)	5′-CGTGTTCCCAG**G**ATCCTCCCTCC-3′	5′-GGAGGGAGGAT**C**CTGGGAACACG-3′	BamHI
C1931T (*h*)	5′-CCCAGAAT**T**CTCCCTCCG-3′	5′-CGGAGGGAG**A**ATTCTGGG-3′	EcoRI
T1942G (*i*)	5′-CCCTCCGA**G**CTCTGTGG-3′	5′-CCACAGAG**C**TCGGAGGG-3′	SacI
T1944G (*j*)	5′-CTCCGATC**G**CTGTGGCGGG-3′	5′-CCCGCCACAG**C**GATCGGAG-3′	PvuI
G1954C (*k*)	5′-GTGGCGG**C**AGCTGAGTTG-3′	5′-CAACTCAGCT**G**CCGCCAC-3′	PvuII
T1969A (*l*)	5′-GTTGGCAG**A**TCTGCTATAAACTG-3′	5′-CAGTTTATAGCAGA**T**CTGCCAAC-3′	BglII
A1787C (*m*)	5′-GCGCTGATAT**C**GGTGACATG-3′	5′-CATGTCACC**G**ATATCAGCGC-3′	EcoRV
C1943A (*n*)	5′-CCTCCGATA**T**CTGTGGCG-3′	5′-CGCCACAGA**T**ATCGGAGG-3′	EcoRV
A2061T	5′-GTCGTGGAGAA**T**TCCACGCCAGC-3′	5′-GCTGGCGTGGA**A**TTCTCCACGAC-3′	EcoRI

[a] For G1505A/T1508C, the nucleotides at 1505 and 1508 were changed from G to A and T to C, respectively, so that restriction enzyme site BamHI was generated. The other mutants were constructed in a similar fashion. *a* to *n* refer to the 14 mutants used in the bottleneck studies.

[b] The letters in boldface type in the primer sequence indicate the mutated nucleotides.

3 sequences (39) was used. Sites with variable nucleotides in the 3′ nontranslated region were chosen for mutation. For the coat protein (CP) coding region, silent mutations were introduced at the third nucleotide in the codon. Site-directed mutagenesis was done by using a standard PCR mutagenesis protocol (36). The mutations were confirmed by sequence analysis of cDNA clones with an ABI 3100 automated sequencing system. The mutants are listed in Table 1. The transcripts of each mutated RNA 3 were generated in vitro and inoculated together with wild-type Fny CMV RNAs 1 and 2. Systemic infection was assessed by the development of symptoms in young uninoculated leaves. Total RNA was extracted from tobacco plant leaves from passage 0 (inoculated with transcripts) and passage 1 (inoculated with extracted viral RNA or plant sap from passage 0) with Tri reagent solution according to the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, Ohio). RNA was used as a template for reverse transcriptase (Superscript, Gibco) with primer 6450 (GGCTGCAG**TGGTCTC CTT**), which is complementary to the 10 terminal nucleotides of Fny CMV RNA 3 (nucleotides in boldface type) and has a linker attached, followed by thermal cycling reactions with primer 6450 and primer 10297 (GAGTCGAGTCATGG ACAAATC), identical to nucleotides 1266 to 1286 of Fny CMV RNA 3 (reverse transcription-PCR [RT-PCR]). This reaction yielded a PCR product of approximately 950 bp. Thermal cycling reaction conditions were as follows: an initial denaturation step at 15 s and 94°C followed by 40 cycles of denaturation at 94°C for 6 s, an annealing step at 60°C for 6 s, and an extension step at 72°C for 25 s with an Idaho Technology Rapid Cycle PCR machine. PCR products were purified by extraction with phenol-chloroform (1:1, vol/vol) saturated with Tris-EDTA buffer followed by ethanol precipitation using standard methods (41). The stability of the mutant viruses was tested by digesting the RT-PCR products from these plants with the enzymes specific for the marker-bearing viruses and sequence analysis of the RT-PCR products at 7 or 14 dpi. Populations were constructed by mixing equal amounts of viral RNA from progeny of each of the individually infected mutants.

Analysis of populations. Plants were inoculated with a mixture of mutants *a*, *b*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *j*, *k*, and *l* (Table 1). Inoculated leaves were excised at 2 dpi, and both the inoculated tissue and surrounding tissues were sampled. Approximately 25 mg of tissue was excised from the 8th and 15th systemic leaves at 10 and 15 dpi, respectively. Total RNA was extracted from sampled tissue and used as a template for RT-PCR as described above. After an estimation of DNA concentration by electrophoresis in agarose gel, the purified PCR products were divided into 9 or 10 aliquots. Each aliquot was then digested with one of the 10 marker restriction enzymes shown in Table 1. Some of the mutants shared the same restriction enzyme sites but at different locations (Table 1). The RT-PCR product from a mixture of the original 12 viral RNAs was used as a control. The presence or absence of each mutant in each inoculated and systemic leaf sample was determined by the presence or absence of its diagnostic bands after digestion and electrophoresis.

Comparison of the populations in different locations of the same systemic leaf. In addition to the experiment described above, a mixture of 12 mutant viruses (*a*, *b*, *c*, *d*, *e*, *f*, *g*, *i*, *j*, *k*, *m*, and *n*) was inoculated onto a small area on the fifth leaf of tobacco plants at the seven-leaf stage. The inoculated leaves were detached at 2 dpi. At 10 dpi, 3 pieces of similarly sized tissue (about 25 mg each) were excised randomly from the eighth leaf (the third leaf above the inoculated leaf) and subjected to mutant virus analysis as described above. In addition, the population comparisons for different locations of the same systemic leaf were carried out for tobacco plants in which the whole fifth leaf was inoculated and the inoculated leaf was detached at 6 dpi.

Data analysis. The analysis of variance was used to test the significance of the mean number of mutants recovered in each experiment (45). A chi-square contingency table was used to test whether all mutants appeared in the systemic leaves with the same frequency (45), thus determining whether their loss during systemic infection occurred stochastically.

The recovery percentage in systemic leaves for each mutant was calculated for each experiment, and the mean recovery percentage of each mutant was obtained by treating each experiment as a replicate. Data were arcsin-square root transformed before analysis of variance. The test of least significant difference was used to compare means among the mutants (45).

RESULTS

Systemic infectivity and stability of the artificial populations. Originally, 15 mutants bearing introduced restriction enzyme site markers in the CP region or the 3′ nontranslated region of Fny CMV RNA 3 were generated by using site-directed mutagenesis (Table 1). All the mutants tested induced systemic mosaic symptoms in tobacco plants. The timing of the appearance and severity of disease symptoms induced by these mutant viruses in tobacco plants were identical to those induced by wild-type Fny CMV (data not shown). Except for the mutant strain A2061T, all mutants were stable through passage 0 and 1, as indicated both by complete digestion of the RT-PCR products from these plants with the enzymes specific for the marker-bearing viruses and by sequence analysis of the RT-PCR products (data not shown). The changed nucleotide T in mutant strain A2061T was reverted to the wild-type A after passage 1, and A2061T was not used further.

Occurrence of a bottleneck during virus infection. Time course experiments showed that 100% of tobacco plants were infected systemically when they were inoculated on local areas of the fifth leaf and the inoculated leaves were detached at 2 dpi. At 4 dpi, the basal portions or the entire seventh and

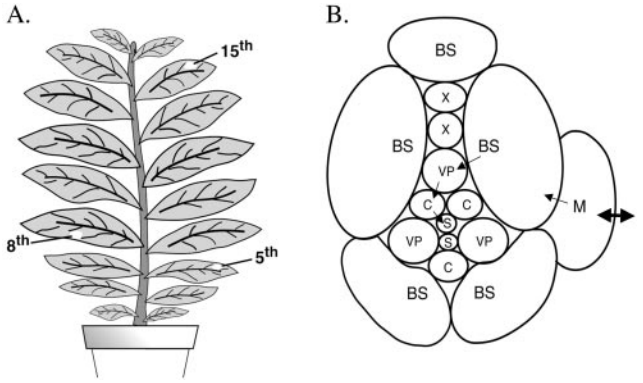

FIG. 1. Schematic representation of plant materials. (A) Tobacco plant showing the location of the inoculated leaf (5th) and the primary (8th) and secondary (15th) systemically infected leaves. (B) Cross-sectional view of a minor vein. The cell-to-cell pathway by which virus is transported via PD is shown by arrows moving from mesophyll cells and passing through BS, VP, and companion cells (C) before entering the sieve elements (S). The PD are abundant in the walls between the mesophyll cells but are limited at the interface of the VP cells and the CC-SE complex. X, xylem.

eighth leaves started to show typical mosaic symptoms, whereas these symptoms were not observed on the sixth leaf. Hence, we designated the seventh and eighth leaves as primary systemically infected leaves. The 15th leaf had not formed when the inoculated 5th leaf was detached at 2 dpi; hence, infection of the 15th leaf represents a secondary infection from the initial systemically infected leaves (Fig. 1A).

To test whether a bottleneck exists during systemic infection, a population of 12 mutants was inoculated onto plants. The population in the inoculated leaves was analyzed by taking samples at 2 dpi as described above. All 12 mutant viruses were detected in the inoculated leaves of all the tested plants from the three experiments (Fig. 2), indicating that all the mutants replicated and moved from cell to cell in the inoculated leaves (nonreplicating RNA inoculum is not detectable after 2 dpi

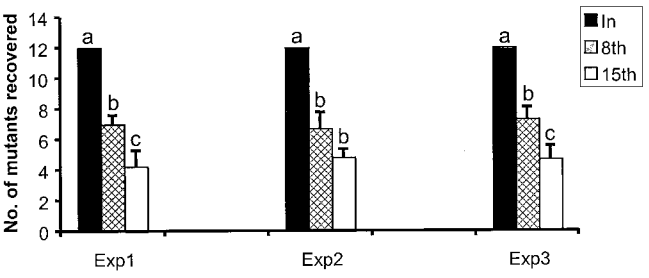

FIG. 2. Reduction in virus population during systemic infection of *Cucumber mosaic virus*. Tobacco plants were inoculated with a mixture of 12 mutant viruses of Fny CMV, and the inoculated leaves and systemic leaves of these plants were harvested at 2, 10 (the 8th leaf), and 15 (the 15th leaf) dpi, respectively. Analysis for the presence of each mutant was carried out by RT-PCR followed by restriction enzyme digestion. Exp1, Exp2, and Exp3 are three independent experiments. In, inoculated leaves; 8th and 15th, systemically infected leaves. All values are expressed as the mean numbers of the recovered mutants ± one standard error. Bars with different letters (a, b, or c) are significantly different from each other ($P < 0.05$), as determined by least significant difference test.

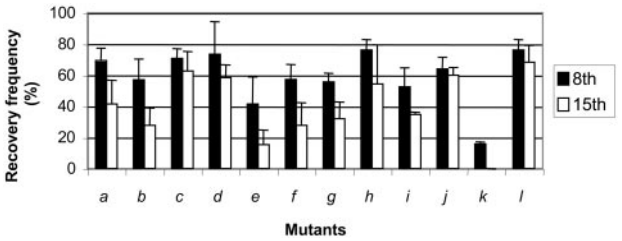

FIG. 3. Recovery frequency of each mutant in systemically infected leaves. The recovery percentage of systemic leaves for each mutant was calculated for each experiment described in the legend to Fig. 2. The mean recovery percentage of each mutant was obtained by treating each experiment as a replicate; *a* to *l* represent the 12 mutants used in this study. All values are expressed as the mean recovery percentages ± one standard error.

[data not shown]). Approximately 25 mg of tissue was excised from the 8th and 15th systemic leaves at 10 and 15 dpi, respectively (Fig. 1A), and used for population analysis. Results from three independent experiments showed that the population size decreased significantly ($P < 0.05$) after the mixed mutant viruses moved from the inoculated leaves to the primary systemic leaves (Fig. 2). The number of mutants recovered from the eighth leaf ranged from 5 to 11, with an average of 7 mutants. The number of mutants recovered from the 15th leaf decreased further as systemic infection progressed, ranging from 2 to 7, averaging 5, and being statistically significant in two out of three experiments ($P < 0.05$) (Fig. 2). In addition, the compositions of the population of mutant viruses in the 8th and 15th leaves analyzed were different from each other (data not shown). No plants were found to have the same mutant populations in this study.

Statistical analyses using a chi-square test of independence demonstrated that 11 of the 12 mutants had the same probability of moving systemically to the eighth leaves. Hence, the elimination of mutants during systemic infection was random, except for mutant *k* (Fig. 3). However, as the virus populations moved further to the 15th leaves, the probabilities of each mutant being present varied to a larger degree. Mutant *k* was never detected, and mutants *b*, *e*, and *f* were recovered with significantly lower frequencies than the others (Fig. 3), indicating that these mutants had a competitive disadvantage when they coinfected tobacco plants. Such differences were undetectable in individual infections in tobacco (data not shown). These results indicated that a significant bottleneck existed during the systemic movement of CMV.

Distribution of genotypes in the population. To further investigate the probable mechanisms involving the genetic bottleneck during the systemic infection, two further experiments were carried out. In the first experiment, a mixture of 12 mutant viral RNAs was inoculated onto a small area of the fifth leaf of each tobacco plant. For these experiments, we replaced the two most frequently recovered mutants from the first set of experiments (*h* and *l*) with two additional mutants (*m* and *n*). The inoculated leaves were detached from the plants at 2 dpi as described above. Three pieces of tissue were excised randomly from the eighth leaf for population analysis at 10 dpi. Consistent with the results described above, all mutants could be detected from the inoculated leaves analyzed, and the pop-

TABLE 2. Changes in virus populations during systemic infection of *Cucumber mosaic virus*

Plant no. and tissue origin[b]	Expt no. and characteristics[a]			
	1		2	
	No. of mutants recovered	Compositions of the population	No. of mutants recovered	Compositions of the population
1, In	12	*abcdefgijkmn*	12	*abcdefgijkmn*
1, A	4	*eijn*	5	*adejn*
1, B	6	*degijk*	5	*cdjkn*
1, C	6	*degijk*	6	*cdegjn*
2, In	12	*abcdefgijkmn*	12	*abcdefgijkmn*
2, A	6	*cdgikn*	5	*acdin*
2, B	5	*cdgkn*	6	*cdgijn*
2, C	5	*cdikn*	6	*cdfgin*
3, In	12	*abcdefgijkmn*	12	*abcdefgijkmn*
3, A	8	*abcdeijn*	8	*acdeijmn*
3, B	8	*abcdjmnj*	8	*acdeijmn*
3, C	8	*abefgijn*	8	*abdeijmn*

[a] For experiment 1, the population of the mutant viruses was inoculated to a small area of the fifth leaf on tobacco at the seven-leaf stage, and the inoculated leaves were detached at 2 dpi. For experiment 2, the population of mutant viruses was inoculated to the whole fifth leaf of tobacco at the seven-leaf stage, and the inoculated leaves were detached at 6 dpi.

[b] 1, 2 and 3 represent the three analyzed plants. In, inoculated leaf; A, B, and C, tissues from different locations of the same systemic leaf (eighth leaf).

ulations were reduced after being moved from inoculated leaves to systemic leaves of all three plants analyzed (Table 2). Interestingly, the population sizes and compositions of most samples varied from the same systemic leaf (Table 2). This result indicated that the mutants were distributed unevenly during systemic movement. In a final experiment, the entire fifth leaf was inoculated and the inoculated leaf was excised at 6 dpi. The populations in different positions of the same systemic leaves were analyzed as was done previously. The population sizes were again reduced after systemic movement, and most of the population compositions in different areas of the same leaf were also different from each other.

DISCUSSION

In this study, we compared the number of genetically marked mutants of CMV present in the inoculated tobacco leaves with those in the primary and secondary systemically infected leaves. Our results showed that the population diversity decreased significantly when the population moved from the inoculated leaves to primary systemic leaves and decreased further as the systemic infection progressed. During this process, the elimination of a majority of the mutants was stochastic. Hence, while the population is affected by selection, there is a significant stochastic bottleneck present during virus movement from the inoculated leaves to uninoculated young leaves by which the CMV population variation is reduced. This finding indicates that systemic movement plays an important role in the genetic structure of RNA virus population in infected plants. This finding also provides a plausible explanation for the observed difference in mutation frequency of CMV in infected tobacco plants versus in protoplasts and the level of genetic variation that was lower than expected (43).

Plant cells are connected by numerous plasmodesmata (PD) through which the virions or nucleic acid of plant viruses can pass (26). Plant viruses encode movement proteins that have the capacity to increase the plasmodesmatal molecular size exclusion limit and facilitate virus movement between cells (26, 28). For a successful systemic infection of a plant virus, the virus must establish an infection in epidermal cells, move through several mesophyll cells followed by vascular bundle sheath (BS) and vascular parenchyma (VP) cells, and then enter into the companion cell-sieve element (CC-SE) complex within the inoculated leaves (Fig. 1B). Once inside the CC-SE complex, the viruses are transported along with the photoassimilates towards sink tissues (8, 21, 28). After viruses reach a systemic leaf, they exit from the phloem cells of major veins, followed by an invasion of BS cells and then mesophyll cells (5, 8, 44).

It is generally accepted that there are restrictions at the interface between the VP and CC-SE complex and that these restrictions may affect systemic movement of the virus in the plant (8, 10, 21, 28, 47). The restriction at the interface of the VP and the CC/SE complex may be associated with the frequency of PD, which are abundant in cell walls between mesophyll cells but are limited at the interface between the VP and the CC/SE complex for plants of apoplastic phloem loaders, including the *Solanaceae* (46). The restriction of virus entry into the phloem may also be associated with different mechanisms that viruses use to move into the vascular tissues (8, 21).

Details of the dynamics of virus loading into and unloading from the phloem are unknown. Inoculation of a small area of the fifth leaf followed by excision at 2 dpi and inoculation of the whole leaf followed by excision at 6 dpi resulted in similar populations in the systemic tissue (Table 2), implying that virus loading into the phloem of the initially infected leaf may occur over a short period of time rather than as a continuous process. It is also possible that infected mesophyll cells exclude superinfection, and this could result in subpopulations in different areas of systemically infected leaves. However, this type of distribution is not seen in inoculated leaves, and given the abundance of PD between mesophyll cells, it seems likely that numerous virions enter a cell simultaneously. Hall et al. (22) found that the coinfection of two related *Wheat streak mosaic virus* strains resulted in spatial subdivisions of virus strains within individual systemic leaves or tillers of plants, with some leaves containing either strain alone and some containing both. An analogous phenomenon was shown recently by fluorescent labeling of potyviruses in both inoculated leaves and systemic leaves of *Nicotiana benthamiana* plants by confocal laser scanning microscopy (9). However, the spatial separation of populations did not occur when two unrelated viruses coinfected the same hosts (9, 22). These results were interpreted as being the result of virus-induced gene silencing at a cellular level. Our findings showed that the compositions of populations in different areas of the same systemic leaf varied from each other (Table 2), indicating that the distribution of the mutants in the systemic leaf occurs in patches. Retaining the inoculated leaf on the plant did not increase the number of mutants recovered from the systemic leaf (Table 2). Hence, the most plausible explanation of the bottlenecks found in this study is that only a subset of genotypes in a virus quasispecies are randomly loaded into vascular tissue in a loading event due to the physical and/or functional restriction of PD at the interface of the

VP and the CC-SE complex. Once this population of genotypes reaches the systemic leaf, they exit and initiate infection. Since different areas of the systemically infected leaves contain different populations, it is plausible that the exit from the phloem is also restricted. Consequently, the populations in the inoculated leaves are segregated and subpopulations are formed in the systemic leaf after the systemic movement.

The presence of bottlenecks associated with human immunodeficiency virus has been deduced by the significant reduction of genetic diversity (19, 24, 29). The most important implication of genetic bottlenecks is genetic drift, which can drive changes in an RNA virus population and the emergence of new virus strains (3, 16, 48). In addition, repeated bottlenecks can result in a loss of fitness, as demonstrated by several experimental systems (4, 12, 31, 32, 49). The influence of bottlenecks on the fitness of the RNA virus population is associated with the bottleneck size. Novella et al. (30) showed that the population will gain fitness in large population transmissions due to competition and optimization of the quasispecies. CMV may be considered an extremely fit virus since it has the largest host range of any known virus, infecting over 1,200 species of plants (13). Clearly, the bottlenecks described in this study are not restrictive enough to cause an overall loss in fitness. They may, however, be sufficient to limit the mutation frequency in systemically infected plants.

Fny CMV did not exhibit any changes in the consensus sequence over multiple passages in several different host species (43). Although this lack of genetic drift under the influence of significant bottlenecks suggests that the majority of individuals do not contain mutations, in some cases (e.g., pepper), every individual virus examined had at least one mutation (43). Hence, negative selection is also an important factor that contributes to the remarkable stability of the CMV genomic sequence.

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