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Genetic bottlenecks during systemic movement of *Cucumber mosaic virus* vary in different host plants

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ABSTRACT

Genetic bottlenecks are stochastic events that narrow variation in a population. We compared bottlenecks during the systemic infection of *Cucumber mosaic virus* (CMV) in four host plants. We mechanically inoculated an artificial population of twelve CMV mutants to young leaves of tomato, pepper, *Nicotiana benthamiana*, and squash. The inoculated leaves and primary and secondary systemically infected leaves were sampled at 2, 10, and 15 days post-inoculation. All twelve mutants were detected in all of the inoculated leaves. The number of mutants recovered from the systemically infected leaves of all host species was reduced significantly, indicating bottlenecks in systemic movement. The recovery frequencies of a few of the mutants were significantly different in each host probably due to host-specific selective forces. These results have implications for the differences in virus population variation that is seen in different host plants.

Introduction

Due to error-prone replication, RNA viruses exist as a genetically diverse population known as a quasispecies (Domingo, 2002). Genetic bottlenecks are random evolutionary events that reduce the genetic diversity of virus populations. Genetic bottlenecks can lead to genetic drift and ultimately to the emergence of new virus strains (Escarmís et al., 1996; Yuste et al., 2000).

Genetic bottlenecks seem to occur frequently during the life cycles of some plant viruses such as *Cucumber mosaic virus* (CMV; Ali et al., 2006; Li and Roossinck, 2004), although infrequently in others (Monsion et al., 2008). These studies have demonstrated bottlenecks experimentally, but the evolutionary effects of genetic bottlenecks during systemic infection of a plant virus in different hosts are largely unknown.

Acute plant viruses must move systemically in their hosts after the initiation of a successful local infection. The systemic movement of plant viruses includes cell-to-cell movement (local spread) from the initially infected cell to the neighboring cells and long-distance movement (vascular-dependent movement) to other tissues of the plant (Nelson et al., 2004). Cell-to-cell movement is achieved through plasmodesmata, intercellular plasma-membrane-lined channels in the cell wall that connect the cytoplasm of neighboring cells and provide passageways for symplastic communication between plant cells. Long-distance movement contains several stages. After inoculation, plant viruses move from the site of initial replication (often epidermal cells) through several layers of mesophyll cells, followed by vascular bundle sheath cells, vascular parenchyma cell, and then a sieve element-companion cell (SE-CC) complex within the inoculated leaves. Viruses from sieve elements are transported along with the photoassimilates toward young (sink) tissues (Nelson and Bel, 1998; Silva et al., 2002). Once viruses reach a systemic leaf, they exit from the phloem and follow the reverse path to reach mesophyll cells in the new leaf (Cheng et al., 2000; Nelson et al., 2004).

In a previous study using an artificial population of CMV consisting of fourteen restriction enzyme marker-bearing mutants, systemic infection in tobacco constituted a significant bottleneck in CMV populations (Li and Roossinck, 2004). To understand the role of genetic bottlenecks in the population structure of CMV, we inoculated twelve of the mutants to seedlings of tomato, pepper, *Nicotiana benthamiana*, and squash, hosts where we previously demonstrated significant differences in the levels of quasispecies variation (Schneider and Roossinck, 2000, 2001). When the systemically infected leaves of these plants were analyzed for the presence of each of the twelve marker mutants, we found significant genetic bottlenecks in the CMV population during the systemic movement in all four host plants, with clear variations among these hosts.

Results

Time course experiment

The time course experiment showed that all four host species used for inoculation were 100% infected systemically with CMV when the

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inoculated leaves were detached at 48, 72, or 96 h. At 24-h detachment of the inoculated leaf, all hosts except tomato were infected systemically. Tomato seedlings did not show systemic symptoms of CMV when the inoculated leaves were detached at 24 h, indicating that the virus takes more than 24 h to move out of the initially infected leaves. Hence we selected 48-h post-inoculation as the time point for harvesting inoculated leaves in subsequent experiments. In previous studies, we found that increasing the time of retention of the inoculated leaf did not change the final population distribution, suggesting that virus movement from the inoculated leaf is essentially a single event (Li and Roossinck, 2004), so we did not detach the inoculated leaves in the subsequent experiments.

Determination of primary and secondary systemically infected leaves

When the first true leaves of tomato and pepper, fifth true leaf of *N. benthamiana*, and a single cotyledon of squash were inoculated with CMV-Fny and detached at 2 dpi, the third true leaves of tomato and pepper, eighth true leaf of *N. benthamiana*, and second true leaf of squash were just emerging (Table 1). At 10 dpi, all these leaves were systemically infected, showing severe symptoms, and were designated as primary systemically infected (PSI) leaves. At 10 dpi, the total number of true leaves in the plants increased to six in tomato, nine in pepper, ten in *N. benthamiana*, and seven in squash. At the time of detachment of the inoculated leaf at 2 dpi, the ninth true leaf in tomato and squash, and eleventh true leaf in pepper and *N. benthamiana* had not formed so these leaves were designated as secondary systemically infected (SSI) leaves.

Identification of bottlenecks during the systemic infection of CMV in different hosts

The mixture of twelve mutant viruses induced systemic symptoms in all inoculated plants of each host**.** Nine plants per host in three independent experiments were analyzed for the mutant population in the inoculated, PSI, and SSI leaves (Supplementary Table 1). The twelve mutants were always detected in the inoculated leaf in each experiment (Fig. 1) indicating that all mutants replicated and moved from cell to cell. However, results from PSI leaves showed that the population of mutant viruses decreased significantly ($P<0.05$) in each host after the mixed CMV mutant population moved from the inoculated to the PSI leaves (Fig. 1).

Similarly, the number of mutants recovered from SSI leaves was further reduced as compared to PSI leaves and was statistically significant (paired *T*-test) in all three independent experiments carried out for each host (Fig. 1b–d) except tomato (Fig. 1a). In tomato, no significant differences were observed between the number of mutants recovered from PSI and SSI leaves in all three experiments using ANOVA-1 (Fig. 1a), the paired *t*-test, multi-ANOVA, and Wilcoxon Signed Ranks non-parametric paired comparisons. However, when the results for all three experiments were pooled there were significant differences between the PSI and SSI leaves of tomato detected by all methods. The total number of mutants recovered from PSI leaves in tomato ranged from one to four with an average of 2.4 ± 1.01 ($P=0.034$, $Z=-2.121$) mutants, in pepper from four to nine with an average of 6.4 ± 1.81 ($P=0.008$, $Z=-2.670$) mutants, in *N. benthamiana* from four to eight with an average of 6.4 ± 1.67 ($P=0.012$, $Z=-2.514$) mutants and in squash from five to eight with an average of 6.1 ± 1.27 ($P=0.007$, $Z=-2.689$) mutants. The number of mutants recovered from SSI leaves decreased further, ranging from one to four, but averaging 1.7 ± 0.97 ($P=0.034$, $Z=-2.121$) in tomato; from one to two, averaging 1.3 ± 0.50 ($P=0.008$, $Z=-2.670$) in pepper; from one to five, averaging 3.7 ± 1.20 ($P=0.012$, $Z=-2.514$) mutants in *N. benthamiana*, and from one to three, averaging 1.78 ± 0.83 ($P=0.007$, $Z=-2.689$) mutants in squash. The differences between PSI and SSI leaves were statistically significant in all three experiments with pepper and squash and in two out of three experiments of *N. benthamiana* (Fig. 1). The composition of the mutant population recovered from individual plants in all hosts were largely different from each other except in some plants of tomato and pepper where identical mutants were recovered, mainly from SSI leaves (Supplementary Table 1).

Table 1
Time course to determine primary and secondary systemically infected leaves.

	Inoculated leaf/total true leaves[a]	No. PSI leaf/total true leaves[b]	No. SSI true leaf/total true leaves[c]
Time of harvest	0 dpi	10 dpi	15 dpi
Tomato	1/3	3/6	9/10[d]
Pepper	1/3	3/9	11/11
N. benthamiana	5/8[d]	8/10	11/11
Squash	C/1	2/7	9/9

[a] Leaf number of inoculated leaf/number of total true leaves at time of inoculation. C, cotyledon.
[b] Leaf number of the PSI/number of total true leaves at time of harvest (10 dpi).
[c] Leaf number of the SSI/number of total true leaves at time of harvest (15 dpi).
[d] Due to the small size of the penultimate leaf, one leaf below was used for SSI in tomato.

Comparison of mutant populations in different hosts

A Chi-square analysis of independence showed that systemic movement of most of the individual mutants was stochastic (Fig. 2a and b), indicating that a bottleneck existed during the systemic movement of CMV from inoculated to PSI leaves in all four host species, and from PSI to SSI leaves in all hosts except tomato. However, the mean recovery frequency of all twelve mutants varied in each host and the recovery frequency of mutants was higher from the PSI leaves (Fig. 2a) as compared to SSI leaves (Fig. 2b). For example, in tomato ten of the twelve mutants were recovered from PSI leaves and all of them had the same probability of moving systemically except mutant *a*, which varied significantly ($P<0.05$). Mutant *a* was recovered in up to 88% of the plants, while mutants *h* and *j* were never detected in PSI leaves. However, in SSI leaves only five mutants (*a*, *c*, *f*, *i*, and *l*) were recovered while the rest were never detected. The recovery frequencies of these five mutants were not significantly different except for mutant *a*, which was recovered in 77% of the SSI leaves.

In pepper, eleven of the twelve mutants were recovered from PSI leaves, but mutant *j* was never detected. The frequency of only two mutants (*c* and *d*) varied significantly ($P<0.05$; 100% each) while the rest of the mutants had no differences in recovery frequency. In SSI leaves, only three mutants (*a*, *c*, and *l*) were recovered. The percent recovery of these mutants was 22% for mutant *a*, 11% for mutant *c*, and 100% for mutant *l*.

In the case of *N. benthamiana*, ten of the twelve mutants were recovered from PSI leaves while mutants *e* and *i* were not detected. The recovery percentage for three mutants (*a*, *d*, and *l*) was up to 100% and was significantly different ($P<0.05$) from the remaining mutants. In SSI leaves, a total of seven mutants (*a*, *c*, *d*, *f*, *i*, *k*, and *l*) were recovered and the percent recovery of mutant *l* varied significantly ($P<0.05$) from the remaining mutants.

In squash, ten of the twelve mutants were recovered from PSI leaves. Mutant *g* and *k* were never detected. Recovery frequency of two mutants (*c* and *l*) was significantly different from the rest of the mutants. In SSI leaves, only five mutants (*a*, *c*, *e*, *l*, and *m*) were recovered. The frequency of mutant *c* recovery was significantly different from the rest of the four mutants.

Hence the recovery frequency of individual mutants varied in different hosts. The frequencies of mutants that were significantly different in each host are probably the result of selective forces in that particular host. However, the remaining mutants have no significant

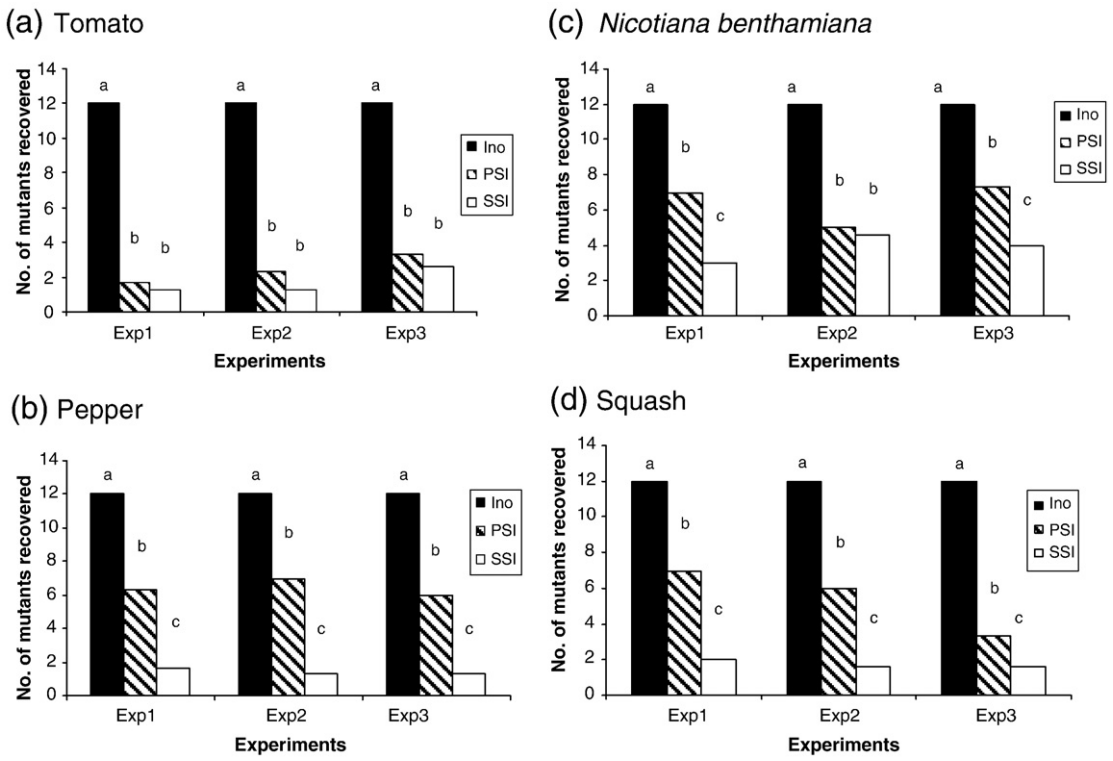

Fig. 1. Reduction in CMV populations during systemic infection from inoculated to primary and secondary systemically infected leaves. Experiments 1, 2, and 3 are three independent experiments containing three plants each per host. All values are the number of recovered mutants. Bars with different letters (a, b, or c) are significantly different from each other within experiment ($P<0.05$), as described in the Materials and methods. Harvested inoculated PSI and SSI leaves were as designated in Table 1. (a) Tomato plants. (b) Pepper plants. (c) *Nicotiana benthamiana* plants. (d) Squash plants.

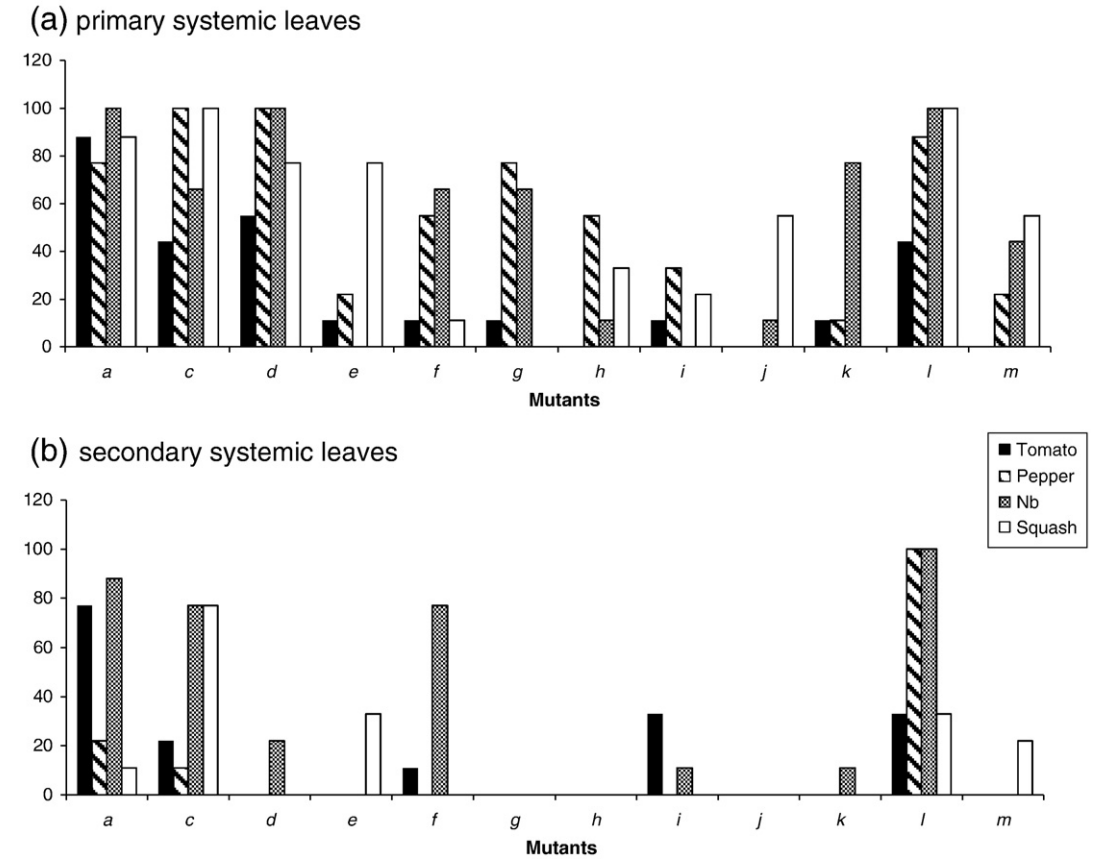

Fig. 2. Summary of the frequency of recovery of each mutant in (a) PSI and (b) SSI leaves of all four hosts. The recovery percentage of each mutant in systemic leaves of each seedling per host was calculated for each experiment as for Fig. 1. Details of the mutants recovered for each plant are in Supplementary Table 1.

differences in their frequencies indicating that they moved stochastically during the systemic movement in these hosts as a result of genetic bottlenecks.

Discussion

In this study, we used an artificial population of twelve CMV mutants and compared the mutant population in the inoculated leaves, and the primary and secondary systemically infected leaves of four different hosts. Our data showed that the number of CMV mutants was significantly reduced when the mutant population moved systemically to the PSI leaves of all the hosts. The mutant population was further reduced during the systemic movement from PSI to SSI leaves in all hosts except tomato, indicating that bottlenecks were found during secondary movement as well. The reduction in the mutant population was largely stochastic in each host, except for a few mutants (*a*, *c*, and *l*) that were apparently affected by selective forces in particular hosts. The bottleneck severity was host specific, with tomato having the most severe bottleneck from inoculated to PSI leaf. However, the number of mutants recovered in the SSI leaves of tomato was not significantly different than in the PSI in individual experiments, suggesting that there was minimal bottleneck effect in the secondary movement of virus in this plant. Since systemic movement of viruses can vary depending on the plant growth conditions, the results within an experiment are more comparable than results when experiments are pooled. In all other species, the apparent bottleneck from the inoculated to the PSI leaf and from the PSI to SSI leaf was similar.

Long-distance movement of plant viruses through phloem includes the loading (entry) of the virus into the phloem of minor veins of the source tissue (sites for phloem loading of photoassimilates), movement through the transport phloem, and unloading (exit) from the phloem into the mesophyll cells in the sink tissue. (Nelson and Bel, 1998; SantaCruz, 1999). The structure of minor veins in leaves of different plant species varies considerably (Oparka and Turgeon, 1999). Three of the host species (tomato, pepper, and *N. benthamiana*) used in this study belong to the same family (Solanaceae), while squash is in a different family (Cucurbitaceae). The number of cells of each minor vein and the minor vein structure are variable among tomato, pepper, and *N. benthamiana* (Ding et al., 1998). Similarly, cell composition and structure of minor veins in squash are different from those in the three plants in the family Solanaceae (Gamalei, 1989, 1991). The frequency of plasmodesmata between different cell types in minor veins varies widely among species (Turgeon and Medville, 2004) and decreases dramatically between the sieve element-companion cell complex and neighboring cells in minor veins (Gamalei, 1989; Turgeon and Medville, 2004; Turgeon et al., 2001). Plasmodesmata are not simple channels in the plant that connect cells. They show a high degree of plasticity and can exist in different, fluctuating states with open, closed, or dilating apertures (Heinlein, 2002). It is likely that differences in apparent bottlenecks among species are due to differences in plasmodesmata structure and biological function and the frequency of plasmodesmata between different cell types in minor veins.

In previous studies in this laboratory, we found significant differences in the levels of population variation in experimentally evolved populations of CMV in different hosts. While mutation frequencies were similar in *N. benthamiana*, squash, and tomato, they were significantly higher in tobacco and higher still in pepper (Schneider and Roossinck, 2001). We hypothesized that differences in mutation rates or in bottlenecks could account for these observations. In this study, although we found significant differences in bottlenecks between tomato and all other hosts tested, this did not correlate with the host-specific differences in mutation frequencies. The high level of population diversity in viruses replicating in protoplasts where there is no cell-to-cell movement, as compared to intact plants (Schneider and Roossinck, 2001), indicates that virus movement plays a significant role in reducing population variation. However, while bottlenecks still undoubtedly contribute to the structure of viral populations, other factors play a more significant role. Differences in polymerase fidelity, or mutation rate, could account for differences in population variation. In a study examining differences in mutation rates in intact plants, we found that the rate of indel mutations was much higher in pepper than in tobacco (Pita et al., 2007), although we do not have data on the substitution rates in these hosts, and substitutions are the predominant type of variation seen in previous viral population studies.

In conclusion, we observed that genetic bottlenecks occur in every host plant tested. However, the severity of the bottlenecks varies and may depend on the structure of the minor veins and plasmodesmata of individual hosts. In addition, the effects of selective forces in the host environment could play a role, particularly in the non-stochastic recovery of certain mutants. Since the mutants do not have alterations in their amino acid sequences, these differences are more likely due to RNA secondary/tertiary structure, or to the RNA–protein interactions that are required for the integrity of the CMV virion (Palukaitis et al., 1992).

Materials and methods

Plant cultures and virus inoculation

Tomato (*Solanum lycopersicum* L. cv Rutgers), pepper (*Capsicum annuum* L. cv Marengo), *Nicotiana benthamiana*, and squash (*Cucurbita pepo* L. cv Zucchini Elite) were germinated and seedlings were used for all experiments in this study. Plants were grown under greenhouse conditions with 26 °C daytime temperature and 20 °C nighttime temperature, and 16-h days. Viral RNAs of twelve CMV mutants (*a*, *c*, *d*, *e*, *f*, *g*, *h*, *i*, *j*, *k*, *l*, and *m*) were mixed in equal concentration and inoculated to seedlings of the host young true leaves or cotyledons as described previously (Li and Roossinck, 2004). Mutants *a*, *c*, *d*, and *m* are silent changes in the coat protein gene; the remaining mutants are found in the 3′ non-translated region of CMV RNA 3. Each mutation consists of a single nucleotide change that introduces a restriction enzyme site, allowing the mutant to be easily monitored using an RFLP-like assay (Li and Roossinck, 2004). Three independent experiments using three plants per experiment were carried out for each of the four hosts used in this study.

Time course experiment

To determine appropriate leaves to use for the primary and secondary systemically infected leaves, we inoculated the first true leaves of tomato and pepper seedlings, the fifth true leaf of *N. benthamiana*, and the cotyledons of squash seedlings with the CMV-Fny strain. The inoculated leaves were detached at 24, 48, 72, and 96 h after inoculation, using two plants per host at each of the four time points. Plants were kept in the greenhouse to observe symptoms of systemically infected leaves at 10 and 15 days post-inoculation (dpi). Two seedlings of each host were kept without detaching the inoculated leaves.

Extraction of total RNA from plants

Total RNA from inoculated leaves and the primary and secondary systemically infected leaves of each plant was extracted from 15 to 25 mg tissue using Tri-Reagent (Molecular Research Centre), according to the manufacturer's instructions. Inoculated leaves were sampled at 2 dpi using both inoculated and surrounding tissues. Tissues from the primary and secondary systemically infected leaves were sampled at 10 and 15 dpi, respectively.

Population analysis

Total RNA extracted from different leaf samples was used as a template for RT-PCR and subsequent enzyme digestion of PCR products as described previously (Ali et al., 2006; Li and Roossinck, 2004).

Data analysis

A multiple analysis of variance (multi-ANOVA), a paired *t*-test, a Wilcoxon Signed Ranks non-parametric test, and a Chi-square test (Mehta and Patel, 1996; Zar, 2010) were used to test the significance of the mean number of mutants recovered from the primary and secondary systemically infected leaves of each host plant. The methods gave the same results. A test of least significant difference was used to compare mean recovery efficiency among the mutants and various hosts. The recovery percentage for each mutant was calculated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.05.017.

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