Aphid vector population density determines the emergence of necrogenic satellite RNAs in populations of cucumber mosaic virus

Mónica Betancourt,† Aurora Fraile, Michael G. Milgroom and Fernando García-Arenal

1Centro de Biotecnología y Genómica de Plantas UPM-INIA and ETSI Agrónomos, Universidad Politécnica de Madrid, Campus de Montegancedo. 28223 Pozuelo de Alarcón, Madrid, Spain
2Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant, Science, Cornell University, Ithaca, NY 14853, USA

The satellite RNAs of cucumber mosaic virus (CMV) that induce systemic necrosis in tomato plants (N-satRNA) multiply to high levels in the infected host while severely depressing CMV accumulation and, hence, its aphid transmission efficiency. As N-satRNAs are transmitted into CMV particles, the conditions for N-satRNA emergence are not obvious. Model analyses with realistic parameter values have predicted that N-satRNAs would invade CMV populations only when transmission rates are high. Here, we tested this hypothesis experimentally by passaging CMV or CMV+N-satRNAs at low or high aphid densities (2 or 8 aphids/plant). As predicted, high aphid densities were required for N-satRNA emergence. The results showed that at low aphid densities, random effects due to population bottlenecks during transmission dominate the epidemiological dynamics of CMV/CMV+N-satRNA. The results suggest that maintaining aphid populations at low density will prevent the emergence of highly virulent CMV+N-satRNA isolates.

Satellite nucleic acids are subviral pathogens that parasitize a helper virus, on which they depend for replication, encapsidation and transmission. Satellite nucleic acids have been used often as model systems to study different aspects of virus biology, including evolution (Simon et al., 2004; Zhou, 2013). Satellite evolution has been approached through both experimentation and the analysis of natural populations of satellite and helper viruses. These studies have addressed fundamental questions in evolutionary biology, such as genetic variation and structure of populations, mechanisms generating genetic variation, mechanisms limiting genetic variation in non-coding nucleic acids, and the role of selection and random drift in virus evolution (García-Arenal & Fraile, 2015). Studies on satellite evolution have concentrated on a few systems, particularly the satellite RNAs (satRNAs) associated with Turnip crinkle virus and with Cucumber mosaic virus (CMV) or with the betasatellite DNAs associated with begomoviruses. These satellites modulate the virulence of their helper viruses, being thus determinants of unique plant diseases, so those studies have also addressed the evolutionary processes that determine disease emergence.

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The conditions leading to the emergence of satRNA-determined plant diseases have been analysed in detail for the satRNA of CMV (family Bromoviridae), which has a tripartite, single-stranded, messenger-sense RNA genome encapsidated in isometric particles. CMV is a generalist parasite infecting more than 1200 species in more than 100 plant families; it is non-persistently transmitted by more than 80 species of aphids (Hemiptera: Aphididae) and through seeds in some host plant species (Jacquemond, 2012). CMV is the helper virus for a satRNA, which is a small (332–342 nt in most variants), non-coding, linear ssRNA. The presence of satRNA leads to a decrease in the accumulation of CMV in the infected plant, and may modulate its virulence depending on the specific strains of CMV and satRNA and the species of host plant infected. Most satRNA variants do not modify, or attenuate, CMV symptoms in most plant species tested; but in tomato, two main phenotypes are distinguished: those that attenuate CMV symptoms (A-satRNAs) and those that cause systemic necrosis (N-satRNAs). SatRNAs occur at low frequencies in most field populations of CMV; however, epidemics of tomato necrosis have been associated with high incidence of N-satRNA (Garcia-Arenal & Palukaitis, 1999; Palukaitis & García-Arenal, 2003).
Conditions for the emergence of N-satRNAs in CMV populations are far from obvious. SatRNAs multiply to high levels in some host plant species, such as tomato, and are efficiently transmitted to CMV progeny (Escriu et al., 2000a, b). In addition, spread of satRNAs would be favoured by the fact that superinfection of plants previously infected by satRNA-free CMV isolates by CMV+N-satRNAs isolates will convert all CMV to CMV+N-satRNA isolates. Accordingly, it was shown that satRNA spreads epidemically as a parasite in the CMV population (Alonso-Prados et al., 1998). However, because satRNA depresses CMV accumulation, and the efficiency of CMV transmission by aphids correlates positively with its accumulation in source plant tissues (Betancourt et al., 2011; Escriu et al., 2000b), the efficiency of transmission of isolates supporting a satRNA may be much less than for satRNA-free isolates. This dynamic represents a trade-off between the efficient within-host multiplication of the satRNA and its between-host transmission, as satRNA multiplication reduces CMV transmission on which the satRNA depends for its own transmission. Also, CMV-satRNA is transmitted by aphids in CMV particles, and CMV aphid transmission results in severe population bottlenecks, with effective founder population sizes of about 1–2, regardless of whether CMV is infected with satRNAs (Betancourt et al., 2008). Thus, random genetic drift is predicted to be important, as it counters any possible selective advantage of N-satRNAs in tomato or other plant species.

Previously, some of us estimated experimentally values for within-host multiplication, competition in mixed infections, and virulence and transmission of CMV isolates free of, or supporting, N- or A-satRNAs (Y, N and A isolates, respectively) in tomato plants. A theoretical model that allowed co-infection of a single host by different isolate types and competition between types with an effect on transmission explained the invasion of the CMV population by N-satRNAs. However, the model predicted that invasion required high transmission rates as a consequence of high densities of aphid vectors (Escriu et al., 2003). These predictions agreed with field observations during tomato necrosis epidemics (Escriu et al., 2000a, 2003). Later, analyses were extended to include another host, melon, in which N-satRNA confers an attenuative phenotype, and in which parameter estimates of multiplication, competition, virulence, and transmission of Y, A and N isolates differ from those in tomato. Model analyses allowing for inter-host transmission in heterogeneous host populations composed of tomato and melon plants also predicted that mixed infections and high transmission rates were required for N-satRNA invasion, and showed that a low frequency of the more competent host, tomato, was sufficient to maintain satRNA-supporting CMV isolates in the less competent host, melon (Betancourt et al., 2013). These analyses predict that high transmission rates are necessary for the invasion of N-satRNA in CMV and for the selection of N isolates. However, the relationship between transmission rates and aphid vector density was not specifically modelled, but simulated with arbitrary values of aphid density, aphid activity and transmission (Betancourt et al., 2013; Escriu et al., 2003). Hence, it is necessary to test model predictions experimentally to determine if indeed high density of aphid populations allows N-satRNAs to emerge and if it results in conditions that counter the population bottlenecks, and the resulting genetic drift, associated with CMV transmission by single aphids. This is the objective of the present report.

To analyse the relationship between aphid population density and N-satRNA emergence, serial passage experiments were performed in which CMV, with or without N-satRNAs, was transmitted by the aphid Aphis gossypii (Glover) within experimental populations of tomato plants (cv. Rutgers). The origin and rearing conditions of the aphid colony have been described previously (Betancourt et al., 2008). All aphid-transmission passages were performed using synchronized populations of apterous females, to minimize differences in transmission efficiency among individuals, as in Fereres et al. (1993). These experiments used strain Fny of CMV (Fny-CMV) and a 1 : 1 : 1 : 1 mixture of four N-satRNA variants collected in the field during an epidemic of tomato necrosis in eastern Spain (variants 89/15.1, 89/24.1, 90/8.2, 91/5.1; Escriu et al., 2000a). Both Fny-CMV and the satRNAs were derived from biologically active full-length cDNA clones (Escriu et al., 2000a; Rizzo & Palukaitis, 1990) and were initially multiplied in Nicotiana tabacum cv. Xanthi-nc plants, from which virions and virion RNA were extracted and used for mechanical inoculation (1 µg virion RNA/plant, for both inocula, with or without satRNAs) of the initial tomato plant population. For serial passage experiments, populations of 40 tomato plants were used. In the initial population (Passage 0, P0), plants were infected with Fny-CMV, with or without satRNAs at different frequencies, and then passages were performed using two aphid population densities, either 2 or 8 aphids per plant (low and high density passages, respectively). For each aphid transmission passage, 2 or 8 aphids were allowed access to each source plant for 5 min, one plant at a time, and were then transferred to target plants in the next passage. Aphids were transferred to randomly chosen plants such that aphids from the same source plant were not transferred to the same target plant, and that 2 or 8 aphids were transferred to each target plant. The order in which source plants were sampled was also randomized. After aphid transfer, each plant was covered with a disposable plastic cup and, after 24 h, was treated with imidacloprid to kill aphids. Inoculations were done when the first true leaf of the tomato plant had expanded totally. Aphid-inoculated plants were maintained in a greenhouse (23 : 18°C day : night temperature, 16 h light) for 15 days, at which time CMV symptoms were apparent, and then were used as sources for the next passage. In Serial Passage Experiment 1 (SPE1), 90% of P0 plants were infected with Fny-CMV, and 10% with Fny-CMV+N-satRNAs; in Serial Passage Experiment 2 (SPE2), 50% of P0 plants were infected with Fny-CMV and 50% with Fny-CMV+N-
satRNAs. Thus, in both experiments, and along passages, plants could have been infected either by CMV or by CMV+N-satRNAs, or plants could have remained uninfected after P0 if aphid transmission failed. For both SPE1 and SPE2, and for each aphid density, three replicated passage lines of 40 tomato plants, were carried out in parallel. For each passage and replicated line, 4 additional plants infected with Fny-CMV, were randomly distributed among the 40 plants of the target population, to control for possible N-satRNA contamination. After each passage population had been used as the source for aphid transmission to the next passage, total RNA was extracted from each plant for detection of CMV and N-satRNA by dot-blot hybridization with specific probes (Betancourt et al., 2011). Passages were stopped when fewer than 10% of plants were infected, so that 4 and 3 passages were done for SPE1 and SPE2, respectively. N-satRNA was never detected in contamination-control plants.

Fig. 1 and Table S1 (available in the online Supplementary Material) show the dynamics of CMV incidence (satRNA-free CMV and N-satRNA+CMV combined) in the tomato populations for all passages. Both in SPE1 and in SPE2, the incidence of CMV-infected plants, with or without N-satRNA, was 100% in P0 and decreased upon passage (Fig. 1a, c). This decrease is easily explained by the efficiency of the aphid transmission process. The frequency of satRNA-free Fny-CMV transmission in tomato plants (infected plants/inoculated plants) was experimentally estimated to be of about 0.40 by the transfer of single aphids from source to target plants, in the same conditions, including the same A gossypii colony (Betancourt et al., 2008). According to Gibbs & Gower (1960), the probability of transmission by a single aphid, p, is \( p = \left(1 - \frac{N}{i}ight)^R \), where \( N \) is the number of inoculated plants, \( R \) the number of infected plants and \( i \) the number of aphids/plant during inoculation, the frequency of transmission, \( R/N \), will increase with increasing numbers of aphids involved in each transmission event, according to the binomial expectation of transmission, thus saturating for high aphid numbers. This relationship explains the faster decrease of CMV incidence when passages were performed with 2 aphids/plant (Fig. 1a) compared with 8 aphids/plant (Fig. 1c). Note that from the estimates of the transmission probability by a single aphid given by Betancourt et al. (2008), the expected probability of transmission of satRNA-free Fny-CMV in one passage would be 0.64 for 2 aphids/plant, and 0.98 for 8 aphids/plant, which would not explain the fast decrease of incidence in high-aphid-density passages. However, the efficiency of transmission varies non-linearly with CMV concentration in source leaves (Betancourt et al., 2011; Escriu et al., 2000b), and CMV accumulation varies among infected plants. Notably, the presence of N-satRNAs severely reduces Fny-CMV accumulation in tomato (Escriu et al., 2000a, 2003), resulting in severe decreases of transmission probability – from 0.40 to 0.11, by a single aphid (Betancourt et al., 2008) – and, therefore, single-passage transmission probabilities of 0.21 and 0.81 for 2 and 8 aphids/plant, respectively. Thus, higher incidences of N-satRNA in our experimental populations should result in faster decreases of CMV incidence upon passage, as indeed is shown by the comparison of the results of SPE1 and SPE2 (Fig. 1a, c).

In SPE1, passages at high-aphid density resulted in the invasion of the CMV population by N-satRNAs, with N-satRNA incidence increasing from 10% to 90% in four passages. By contrast, N-satRNAs disappeared from the CMV population after the first passage at low aphid density (Fig. 1b). These results are in complete agreement with model analyses, which predicted that satRNA invasion of CMV populations required high aphid transmission rates, which are correlated with high aphid densities (Betancourt et al., 2013; Escriu et al., 2003). At low-aphid density, N-satRNAs are lost from the CMV population because transmission is less efficient; transmission from satRNA-free Fny-CMV-infected plants is more efficient than from Fny-CMV+N-satRNAs-infected plants (see above), and because transmission of satRNAs to the CMV progeny, being high (=80%), is not 100% (Escriu et al., 2000a). Results of SPE2 initially also agreed with those of SPE1, with model predictions and with previous results about CMV and satRNA transmission; passaging with high aphid density resulted in the increase of N-satRNA incidence from 50% to 90% in two passages, whereas this was not the case at low aphid density (Fig. 1d). Note, however, that the variance of N-satRNA incidence was much higher at low aphid density than at high aphid density (standard errors being 2–8 times higher, Fig. 1b, d and Table S1). This result indicates low predictability of N-satRNA incidence, and is in agreement with the random transmission of N-satRNA to new plants when transmission efficiency is lower. Stochasticity is also evident in the sudden decrease of N-satRNA incidence in high-aphid-density P3 and in its sudden increase at low-aphid-density P3. If the evolution during SPE2 of Fny-CMV and of N-satRNA incidence is compared, it can be concluded that the very low Fny-CMV incidence (<5%) at low-aphid-density P2, with 50% N-satRNA incidence, may cause a random increase of N-satRNA incidence because so few infected plants serve as sources for transmission to the next generation. Similarly, the low incidence of Fny-CMV at high-aphid-density P2 (20%) with 90% N-satRNA incidence will result in a random change of N-satRNA incidence associated with low transmission efficiency.

Thus, our results show that, as predicted by model analyses, and due to the complexity of the CMV, satRNA and host plant interactions, high aphid densities are required for N-satRNA invasion of the CMV population. This is because the exponential relationship between transmission frequency and number of aphids per transmission event determines the relative fitness of CMV isolates supporting N-satRNAs and satRNA-free isolates: that difference in fitness, expressed in terms of new infections per infected plant, is maximal for 2–4 aphids/plant, and decreases with increasing number of aphids (Escriu et al., 2003). In addition to providing experimental evidence for model
predictions, the results presented here show that at low aphid densities random effects due to population bottlenecks during CMV transmission (Betancourt et al., 2008) dominate the epidemiological dynamics of CMV populations, and N-satRNAs can only invade the CMV population at high aphid densities, which is favoured by N-satRNA multiplying to high densities in tomato plants, and by the efficient transmission of N-satRNA to CMV progeny (Escriu et al., 2000a). Finally, our results are also relevant to understanding and preventing N-satRNA emergence: the control of aphid vector populations by the use of insecticides, or by other means, will result in a decrease of both CMV incidence and CMV virulence by preventing the emergence of highly virulent, N-satRNA-supporting isolates.

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