

Estimation of the number of virus particles transmitted by an insect vector

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Plant viruses are submitted to narrow population bottlenecks both during infection of their hosts and during horizontal transmission between host individuals. The size of bottlenecks exerted on virus populations during plant invasion has been estimated in a few pathosystems but is not addressed yet for horizontal transmission. Using competition for aphid transmission between two *Potato virus Y* variants, one of them being noninfectious but equally transmissible, we obtained estimates of the size of bottlenecks exerted on an insect-borne virus during its horizontal transmission. We found that an aphid transmitted on average 0.5–3.2 virus particles, which is extremely low compared with the census viral population into a plant. Such narrow bottlenecks emphasize the strength of stochastic events acting on virus populations, and we illustrate, in modeling virus emergence, why estimating this parameter is important.

aphid | genetic drift | population bottleneck | potyvirus | nonpersistent virus

Horizontal transmission to switch host individuals, which is a critical step in the life cycle of parasites, is ensured by specific biological vectors for many plant and animal viruses. Two major categories of plant viruses, persistent and nonpersistent, can be distinguished according to their transmission mode. Nonpersistent viruses have very short retention times in their vector, usually <12 h. Acquisition and inoculation access periods (AAP and IAP, respectively) are short for these viruses (<5 min). Nonpersistent viruses are not simply transmitted by pure mechanical processes (the vector acting as a “dirty needle”), but a high degree of specificity exists between vector and viral components (1). This kind of virus transmission, largely shared by plant viruses, seems absent among animal viruses. An exception could be *Lumpy skin disease virus* (family *Poxviridae*, genus *Caprifox*), which is transmitted by the mosquito *Aedes aegypti* in a more complex way than by a “dirty needle” (2), but information on the characteristics of the virus–vector interaction is still lacking.

Persistent viruses, in contrast, require longer AAP and IAP, and retention of the virus can last from \approx 12 h to the lifetime of the vector. Between acquisition and inoculation, a latent period of one to several days is necessary for persistent viruses to be transmitted, whereas it is not for nonpersistent ones.

Aphids are the most widespread plant virus vectors and can transmit both persistent and nonpersistent viruses. More than 200 plant virus species are transmitted nonpersistently by aphids. Virus particles inoculated to plants are previously retained either directly or indirectly in the mouthparts of aphids. In the case of indirect interaction, binding of virus particles to the aphid mouthparts is mediated by one or more viral-encoded proteins, the helper component (3). For example, aphid transmission of viruses from the genus *Potyvirus* involves the helper component-proteinase (HC-Pro) protein, which plays the role of a “molecular bridge” by its interactions with the virus coat protein (CP) and with the vector’s stylet (4). As a consequence, only mutations in the HC-Pro and CP of potyviruses have been shown to affect their transmissibility by aphids. The aphid receptors allowing

retention and inoculation of nonpersistent viruses are unknown but are presumably localized at the distal tip of the stylet bundle (5, 6). Virus acquisition and inoculation occur during the brief (\approx 10 s) intracellular punctures (or probes) exerted by aphids into epidermal or mesophyll plant cells to select their hosts.

The effects of horizontal transmission by vectors on the evolution of plant virus populations are largely unknown. Vectors are presumed to impose both selective and bottlenecks effects on virus populations. The selective impact of aphids was clearly demonstrated by relaxing the aphid transmission constraint on plant virus populations, during series of mechanical inoculations in the laboratory (1). A large number of virus variants issued from these experiments were shown to be poorly or non transmissible by vectors. Differential transmission efficiency of virus variants between different aphid species was also shown experimentally (7) and could be a major evolutionary constraint on some virus populations (8). Genetic drift was also demonstrated recently by the stochastic extinction of genotypes in CMV populations after nonpersistent aphid transmission due to narrow bottlenecks during transmission (9). Besides these qualitative results, estimating the size of the bottlenecks exerted by vectors is crucial to quantify their consequences on virus evolution.

Very few studies have attempted to estimate the size of bottlenecks exerted on plant virus populations, either persistent or nonpersistent, during transmission by vectors. Pirone and Thornbury (10) estimated the number of particles of *Tobacco etch virus* or *Tobacco vein mottling virus* (genus *Potyvirus*) in aphids after acquisition of purified, radioactively labeled virus. In their experiments, individual aphids that succeeded in infecting a plant after the transmission procedure acquired from 15 to 20,760 potyvirus particles. Given the relatively small number of aphids analyzed and given such a huge range of variation, it remains important to get a more precise estimate of the average number of acquired virus particles. In addition, these figures certainly overestimate the number of transmitted virus particles because they include virions that did not contribute to plant infection, i.e., (i) those that were ingested, (ii) the stylet-borne virions that will not be inoculated to the plants, and (iii)

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Abbreviations: PVY, *Potato virus Y*; AAP, acquisition access period; IAP, inoculation access period; HC-Pro, helper component-proteinase; λ_a , total number of PVY particles transmitted by an aphid to a plant after *in vitro* acquisition; λ_s , total number of PVY particles transmitted by an aphid to a plant after *in planta* acquisition; κ , number of aphid probing punctures required to inoculate the acquired virus particles; λ_p , number of PVY particles required to infect a plant; Vir, PVY variant SON41; Avir, PVY variant SON41xVPgLYE84.2.

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stylet-borne virions that will be inoculated to the plants but that are unviable because of deleterious mutations.

In the present article, we analyze results of competitions for aphid transmission between two *Potato virus Y* (PVY; genus *Potyvirus*) variants to get an estimate of the size of population bottlenecks during transmission. By-products of our analyses are estimates of two other important epidemiological parameters: the number of virus particles required to infect a plant and the number of probes made by aphids to inoculate the acquired virus particles.

Results

Evidence for Population Bottleneck During PVY Transmission by Aphids Revealed by Competition Between Virulent and Avirulent Virus Variants. In our experiments, virus acquisition was carried out by allowing aphids to feed on artificial mixtures of two PVY variants (SON41 and SON41xVPgLYE84.2 (11), named in the following Vir and Avir, respectively) in different relative proportions in an *in vitro* device (12). The two PVY variants differ only by 14 nucleotide substitutions (corresponding to five putative amino acid substitutions) in the virus protein genome-linked (VPg) cistron. These substitutions determine the ability to infect [called virulence in the field of plant pathology (13)] pepper (*Capsicum annuum* L.) plants carrying the *pvr2¹* recessive resistance allele (11). The Vir variant is virulent toward *pvr2¹* whereas the Avir variant is not (i.e., it is avirulent). However, both Vir and Avir are virulent toward the *pvr2⁺* allele. After feeding, aphids were transferred onto pepper plants for inoculation, and the number of infected plants was recorded for each Vir:Avir ratio [Table 1, columns I–VIII, XI, and XII; and [supporting information \(SI\) Fig. 3](#)].

When a single aphid per plant was used for PVY transmission, the infection frequency was low and decreased rapidly as the proportion of the Vir component decreased. Consequently, the experiment was not much informative and confidence intervals of estimated parameters were excessively large (data not shown). We therefore used two aphids per plant for inoculation in the following experiments. Comparing the infection frequency of “Yolo Wonder” plants (*pvr2⁺/pvr2⁺*) inoculated after acquisition from a medium containing only the Vir or the Avir variants with identical concentrations showed no significant differences ($0.35 < P \text{ value} < 0.69$, depending on the experiments; Fisher’s exact test; Table 1, columns XI and XII). We may consequently consider that the two PVY variants share the same aphid transmission efficiency.

A marked decrease of the frequency of PVY-infected “Yolo Y” (*pvr2¹/pvr2¹*) plants was observed as the relative proportion of the Vir component decreased in the acquisition medium (Fig. 1). This decrease was not due to lower absolute concentrations of the Vir component in the acquisition media. Indeed, a strong competition effect is revealed if we compare the frequencies of “Yolo Y” plants infected from a medium containing 10% of Vir to those obtained from a ten fold dilution of the Vir variant in buffer. The absolute concentration of the Vir component is the same (3.5 ng/μl) in both acquisition media, but the infection frequency is drastically reduced in case of competition for transmission between the two PVY variants (P values $< 3 \times 10^{-4}$, Fisher’s exact test; Table 1, columns II and VII). This result indicates that there is a limited number of binding sites in the aphid stylet and that the number of Vir particles transmitted to the plants is drastically reduced when the Avir component is also present in the acquisition medium, therefore revealing that a bottleneck affected the total PVY population.

Estimation of the Size of Bottlenecks Affecting PVY Populations During Transmission. A simple stochastic model was developed to estimate the size of bottlenecks affecting PVY populations during aphid transmission from the experiments described

Table 1. Aphid transmission of two variants of *Potato virus Y* (Vir and Avir) to pepper plants

| Exp. no. | Plant stage ^a | Infection frequency in “Yolo Y” (<i>pvr2¹/pvr2¹</i>), <i>in vitro</i> acquisition [†] | | | | | | | | Infection frequency in “Yolo Y” (<i>pvr2¹/pvr2¹</i>), <i>in planta</i> acquisition [‡] | | | | Infection frequency in “Yolo Wonder” (<i>pvr2⁺/pvr2⁺</i>), <i>in vitro</i> acquisition [†] | | | |
|----------|--------------------------|--|---------------|---------------|----------------|-----------------|----------------|---------------|--------------|---|----------------|---------|---------------|---|---------|----------|--|
| | | 1.00 [§] I | 1.00 II | 0.75 III | 0.50 IV | 0.40 V | 0.25 VI | 0.10 VII | 0.00 VIII | 1.00 IX | 1.00 X | 0.00 XI | 1.00 XII | 0.00 XI | 0.00 XI | 1.00 XII | |
| 1 | 18 days | 51/95 (53.7%) | 40/61 (65.6%) | 49/92 (53.3%) | 48/94 (51.1%) | — ^{††} | 22/95 (23.1%) | 14/90 (15.6%) | 0/87 (0.0%) | 76/108 (70.4%) | — | — | 43/72 (59.7%) | 56/90 (62.2%) | — | — | |
| 2 | 18 days | 58/102 (56.9%) | 50/95 (52.6%) | — | 58/128 (45.3%) | 41/126 (32.5%) | 21/120 (17.5%) | 12/130 (9.2%) | 0/101 (0.0%) | 50/72 (69.4%) | — | — | 30/47 (63.8%) | 53/90 (58.9%) | — | — | |
| 3 | 25 days | 8/34 (23.5%) | 11/45 (24.4%) | — | 7/42 (16.7%) | 5/43 (11.6%) | 1/40 (2.5%) | 0/43 (0.0%) | — | 25/61 (41.0%) | — | — | — | 9/42 (21.4%) | — | — | |
| 4 | 18 days | — | — | — | — | — | — | — | — | 59/120 (49.2%) | 30/100 (30.0%) | — | — | — | — | — | |

Absolute PVY concentration in the acquisition medium (Vir plus Avir) was 35 ng/μl in columns I, III–VIII, XI, and XII; it was 3.5 ng/μl in column II.

Time allowed for virus inoculation was 16 h for columns I–IX, XI, and XII; one probe only was allowed for column X.

[†]Inoculations performed with two aphids per plant.

[‡]Inoculations performed with one aphid per plant after acquisition from “Yolo Wonder” leaves.

[§]Proportion of the Vir PVY variant in the acquisition medium relative to the total PVY population (Vir plus Avir).

^aDays after sowing.

|| Infected/inoculated plants.

†† Not tested.

estimates ($N < 10$), extinction of Vir viruses is nearly certain only for high fitness costs ($s > 0.1$). Aggregation of Vir and Avir particles counterbalances the effect of s by increasing the strength of stochastic effects, i.e., for a given s , N_1 is increased (Fig. 2A). In the (s, N) plane, the area where the extinction of Vir viruses is nearly certain is consequently reduced (Fig. 2C).

Regarding the number of transmission events required to achieve extinction of the Vir viruses, the mean of τ ($\bar{\tau}$) increases linearly with N (in a x -log, y -log scale) in the absence of any fitness cost of virulence ($s = 0$). When s increases, the plot of $\bar{\tau}$ versus N curves downwards with increasing values of N and the larger N , the stronger the effect of selection if virulence has a cost because genetic drift is reduced (Fig. 2D). However, as previously, a threshold value N_2 of N appears below which $\bar{\tau}$ depends mainly on N and little on s . There, whatever s , plots of $\bar{\tau}$ versus N are all merged. For a given fitness cost s , the ratio $\bar{\tau}(s, N)/\bar{\tau}(0, N)$ is thus close to one when $N < N_2$ (Fig. 2D). In the (s, N) plane, the area where N is the major determinant of $\bar{\tau}$ [$\bar{\tau}(s, N)/\bar{\tau}(0, N) > 0.9$] defines a lower left triangle, N_2 decreasing linearly with s (SI Fig. 4). Importantly, in the range of our estimates of N ($N < 10$), $\bar{\tau}$ is mostly determined by N in a large range of s (10^{-4} , 0.03). Even moderate rates of aggregation of Vir and Avir particles within plants decreased $\bar{\tau}$, especially for high N values (Fig. 2D). In addition, virus aggregation reduced the effect of s by enlarging the area where N is the major determinant of $\bar{\tau}$ for higher N values (100, 1,000), N_2 decreasing only slightly for $N > 100$ (SI Fig. 4).

Discussion

We obtained the first estimates of the size of the bottleneck acting on plant virus populations during vector transmission. Occurrence of bottlenecks during nonpersistent aphid transmission was recently demonstrated for CMV (9), but the authors did not provide any estimate for the size of bottlenecks. In their experiments, artificial populations of 12 virulent variants of CMV were used in acquisition media for aphid transmission. The decrease of the number of CMV variants detected in inoculated plants evidenced the bottleneck effect of aphids during virus transmission. However, it seems difficult from these experiments to untangle the bottleneck effect of aphids during transmission from the bottleneck effect during plant invasion, which was shown to be quite severe (15–17). In contrast, our analysis of the bottleneck effect exerted by aphids was not based on the loss of richness of virus populations between the inoculum and the inoculated plants, but on the competition between two virus variants, one of them being noninfectious (avirulent). As a consequence, genetic drift potentially acting on viral populations during plant infection did not affect our parameter estimates. Our estimates of the size of bottlenecks during potyvirus transmission by aphids were much smaller than those of Pirone and Thornbury (10), even if the comparison is partial because no average values were given in their study. Reasons of such differences hold certainly for the fact that their estimates included all of the virus particles present in the aphid body, whereas only those retained in the aphid stylet bundle can be efficiently transmitted to the plants. Combined with the high diversity of virus populations owed to their high mutation rates, a consequence of such narrow bottlenecks is certainly an intense genetic drift, because the huge majority of the virus variants will have no progeny after aphid transmission.

Genetic drift seems to affect frequently and severely plant virus populations. Indeed, recent studies have demonstrated that drift occurs intensely during plant invasion by viruses. Only around 10 virus particles contributed to the systemic infection of plants after mechanical inoculation (15, 17) although several hundreds initiated an infection (17). We show here that, in addition to genetic drift experienced by viral populations within plant hosts, extremely narrow bottlenecks also shape plant virus

populations during nonpersistent aphid transmission. These repeated and narrow bottlenecks during virus epidemics may have several important consequences on virus evolution, such as decreases of fitness (18, 19), of (broad-sense) virulence (20), and of the capacity to respond to natural selection (21) and could also contribute to the differentiation of plant virus populations at the field or regional scales. Also, the quasi-species model of viruses, which assumes an absence of genetic drift during the evolution of virus populations, could be irrelevant for some plant viruses because of the drastic effect of bottlenecks (22).

From an applied perspective, deriving estimates of parameters central to virus evolution is of major interest for modeling disease emergence and is an important issue in agricultural production as illustrated by the breakdown of resistance genes by virulent virus isolates (23). Mathematical models are key tools here in allowing to describe the interplay between evolutionary and epidemiological processes acting on virus population both at the within-host and at the plant population scales (24, 25). Such approaches are not classic in plant virus epidemiology, where models are most often focused on the spatiotemporal dynamics of epidemics at the field scale (24). Our simulation model illustrates nicely that bridging evolutionary and epidemiological processes which can operate at different scales is required to understand the emergence of virulent virus variants. It explores how describing the within-host dynamics of virus population (fitness cost s of virulence and heterogeneity θ of distribution of virus variants) and the between-host transmission (number N of virus transmitted by aphids), allow to predict the emergence of a virulent virus. Interestingly, we found that, in the range of our estimated N values, emergence of a virulent virus variant, although highly dependent on N , was rather insensitive to s , highlighting the poor responsiveness of the virus population to selection mentioned above. The insensitivity to s was still enhanced by increasing values of θ . θ is an important parameter to take into account for plant viruses, because compartmentalization of virus variants within plants was frequently observed (15, 26, 27) and may be the consequence of genetic drift that occurred at the plant tissue level during plant infection.

As a conclusion, we showed that estimating the number of virus particles involved in horizontal transmission is crucial for predicting the emergence of virus variants and we provided such estimates for an aphid-transmitted nonpersistent plant virus. Our experimental approach based on the competition between a virus and a noninfectious but equally transmissible variant, as well as our stochastic model, could be used for other kinds of viruses, provided the availability of an efficient *in vitro* acquisition protocol and of a suitable host resistance system.

Methods

The two PVY variants Vir and Avir [called SON41 and SON41xVPgLYE84.2, respectively, in Moury *et al.* (11)] were purified separately from infected tobacco (*Nicotiana tabacum* cv. Xanthi) plants using a modified version of method 1 of Moghal and Francki (28), where chloroform and carbon tetrachloride in the extraction buffer were replaced by 1,1,2-trichlorotrifluoroethane. Their concentrations were estimated with double-antibody sandwich ELISA (DAS-ELISA) dilution-absorbance curves as in Moury *et al.* (11) by comparison with a reference purified stock of the Vir variant of known concentration. A virus-free fraction of Xanthi plants infected by the Vir variant containing 20% (wt/vol) sucrose was obtained as in Thornbury *et al.* (29) and added to the acquisition medium. This fraction contained the PVY HC-Pro, a protein required for aphid transmission.

Wingless aphids (*Myzus persicae* Sulzer) reared on *C. annuum* “Yolo Wonder” plants in a growth chamber were starved during 3–4 h prior acquisition. They were allowed to feed during 5–6 min in a medium containing the purified PVY variants in various

relative proportions and half volume of the solution containing HC-Pro through a stretched Parafilm membrane in a similar way as in Govier *et al.* (12). This AAP was shown to be optimal for transmission (not significantly different from a 1- to 2-min AAP), whereas transmission efficiency decreased significantly for a 10- to 11-min or 15- to 16-min AAP (data not shown). The total (Vir plus Avir) PVY concentration was identical for all of the artificial acquisition media (35 ng/ μ l), except one series of inoculations performed after acquisition from a medium containing 3.5 ng/ μ l PVY (Table 1, column II). Fed aphids were transferred with a paintbrush to *C. annuum* “Yolo Y” (*pvr2¹/pvr2¹*) or “Yolo Wonder” (*pvr2⁺/pvr2⁺*) seedlings at the cotyledon or one-expanded-leaf stage (18 or 25 days after sowing, respectively), depending on the experiment. “Yolo Wonder” and “Yolo Y” are nearly isogenic inbred lines with different alleles at the *pvr2* locus (30). Inoculations were performed with one or two aphids per plant, depending on the experiment, which were maintained on a single cotyledon of the plants overnight and treated with an insecticide. Additional experiments were performed by using apical leaves of “Yolo Wonder” plants infected

with the Vir variant for acquisition. In one of these latter experiments, aphids were allowed to perform only one probe during the IAP as assessed by the observation of their behavior under the microscope (immobilization of aphids during a short period of \approx 10 s with typical posture of legs and antennae). Afterward, they were immediately withdrawn from the plants with a paint brush. After the IAP, the plants were maintained in greenhouse conditions for one month. Their infection status was then checked by symptom reading, and DAS-ELISA was performed on apical, uninoculated leaves.

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1. Ng JCK, Falk BW (2006) *Annu Rev Phytopath* 44:183–212.
2. Chihota CM, Rennie LF, Kitching RP, Mellor PS (2001) *Epidemiol Infect* 126:317–321.
3. Froissart R, Michalakakis Y, Blanc S (2002) *Phytopathology* 92:576–579.
4. Pirone T, Blanc S (1996) *Annu Rev Phytopath* 34:227–247.
5. Wang RY, Ammar ED, Thornbury DW, Lopez-Moya JJ, Pirone TP (1996) *J Gen Virol* 77:861–867.
6. Martín B, Collar JL, Tjallingii WF, Fereres A (1997) *J Gen Virol* 78:2701–2705.
7. Perry KL, Zhang L, Palukaitis P (1998) *Virology* 242:204–210.
8. Moury B (2004) *Mol Biol Evol* 21:1602–1611.
9. Ali A, Li H, Schneider WL, Sherman DJ, Gray S, Smith D, Roossinck MJ (2006) *J Virol* 80:8345–8350.
10. Pirone TP, Thornbury DW (1988) *Phytopathology* 78:104–107.
11. Moury B, Morel C, Johansen E, Guilbaud L, Souche S, Ayme V, Caranta C, Palloix A, Jacquemond M (2004) *Mol Plant Microbe Interact* 17:322–329.
12. Govier DA, Kassanis B, Pirone TP (1977) *Virology* 78:306–314.
13. Shaner G, Stromberg EL, Lacy GH, Barker KR, Pirone TP (1992) *Annu Rev Phytopathol* 30:47–66.
14. Watson MA (1972) in *Principles and Techniques in Plant Virology*, eds Kado CI, Agrawal HO (Van Nostrand Reinhold, New York), pp 131–167.
15. French R, Stenger DC (2003) *Annu Rev Phytopath* 41:199–214.
16. Li H, Roossinck MJ (2004) *J Virol* 78:10582–10587.
17. Sacristán S, Malpica JM, Fraile A, García-Arenal F (2003) *J Virol* 77:9906–9911.
18. Chao L (1990) *Nature* 348:454–455.
19. Duarte E, Clarke D, Domingo E, Holland J (1992) *Proc Natl Acad Sci USA* 89:6015–6019.
20. Bergstrom CT, McElhany P, Real LA (1999) *Proc Natl Acad Sci USA* 96:5095–5100.
21. Hill WG, Rasbash J (1986) *Genet Res* 48:125–131.
22. French R, Stenger DC (2005) *Virology* 343:179–189.
23. Lecoq H, Moury B, Desbiez C, Palloix A, Pitrat M (2004) *Virus Res* 100:31–39.
24. Scherm H, Ngugi HK, Ojiambo PS (2006) *Eur J Plant Pathol* 115:61–73.
25. Jeger MJ, Holt J, van den Bosch F, Madden LV (2004) *Physiol Entomol* 29:291–304.
26. Dietrich C, Maiss E (2003) *J Gen Virol* 84:2871–2876.
27. Jridi C, Martin JF, Marie-Jeanne V, Labonne G, Blanc S (2006) *J Virol* 80:2349–2357.
28. Moghal SM, Francki RIB (1976) *Virology* 73:350–362.
29. Thornbury DW, Hellmann GM, Rhoads RE, Pirone TP (1985) *Virology* 144:260–267.
30. Cook AA (1961) *Phytopathology* 51:550–552.